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Mechanisms Of Host Cell Control Over Coronavirus Replication Within Glial Cells From The Nervous System Of Rodents

Greame A. Wilson

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MECHANISMS OF HOST-CELL CONTROL OVER CORONAVIRUS REPLICATION WITHIN GLIAL CELLS FROM THE NERVOUS SYSTEM OF RODENTS

by

Greame A. R. Wilson
Department of Microbiology and Immunology

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Abstract.

In vitro primary cultures of glial cells from neonatal rats and mice were established and utilized in an effort to better characterize the virus-cell interactions and host-cell mechanisms controlling coronavirus (CV) infections within the nervous system of rodents.

Primary cerebral explant cultures from mice, containing oligodendrocytes and astrocytes, when infected with CV-JHMV and MHV_3 demonstrated no discrimination in cell tropism with both viruses replicating in either cell-type. Induction of oligodendrocyte differentiation with dbcAMP treatment resulted in a profound suppression of CV replication consistent with previous findings on JHMV replication in differentiated rat oligodendrocytes. A possible mechanism for this CV restriction may involve an inhibition of nucleocapsid protein (NC) dephosphorylation occurring within host-cell endosomes during an early stage of virus infection. Preparations of the regulatory subunit of cAMP-dependent protein kinase type 1 (R_1), found in elevated levels in differentiated oligodendrocytes, inhibited an endosomal phosphoprotein phosphatase, which dephosphorylates the NC protein, thereby controlling this enzymes activity.

Additional studies utilizing primary glial cultures from neonatal SJL mice documented a genetically determined host-cell mechanism controlling CV serotype specific infection. Mixed glial cultures from SJL mice restricted the replication of CV-JHMV and A59 but fully supported the replication of the highly sero-related strain MHV_3. A comparative analysis of the infectious process demonstrated no difference in the early events of virus infection from adsorption to genome transcription but localized the restriction to the intercellular spread or dissemination of the infection from initial foci.

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ABBREVIATIONS

ATP	adenosine triphosphate
BME_{10}	basal medium Eagle's with 10% fetal bovine serum
BSA	bovine serum albumin
C	degrees Celcius
cAMP	adenosine 3':5'-cyclic monophosphate
cDNA	complementary deoxyribose nucleic acid
Ci	Curies
cm	centimeter
CNPase	2':3'-cyclic nucleotide-3'-phosphohydrolase
CPE	cytopathic effect
cpm	counts per minute
CV	coronavirus(es)
Da	Daltons
dATP	deoxyadenosine triphosphate
dbcAMP	N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxygyanosine triphosphate
DNA	deoxyribose nucleic acid
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis (beta-aminoethyl ether) - N,N,N,N, tetraacetic acid

FBS	fetal bovine serum
g	gram(s)
GC	galactocerebroside(s)
GFAP	glial fibrillary acidic protein
G>M-FITC	goat anti-mouse fluorosceneisothiocyanate
G>Ra-RITC	goat anti-rabbit rhodamineisothiocyanate
hr	hour(s)
IgG	immunoglobulin G
JHMV	mouse hepatitis virus type 4
l	liter
M	molar
MBP	myelin basic protein
uCi	micro Curies
2-ME	2-mercaptoethanol, (beta-mercaptoethanol)
MES	(2[N-morpholino] ethanesulfonic acid)
mg	milligram(s)
ug	microgram(s)
MHV	mouse hepatitis virus
MHV_3	mouse hepatitis virus type 3
min	minute(s)
ml	milliliter(s)
ul	microliter(s)
mM	millimolar
mm	millimeter(s)
um	micrometer(s)
umoles	micromoles

M>MHV	mouse anti-mouse hepatitis virus
MW	molecular weight
N	normal:as in normality of a solution
NC	nucleocapsid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFU	plaque forming units
PPPase	phosphoprotein phosphatase
PK_I	cAMP dependent protein kinase 1
PK_2	cAMP dependent protein kinase 2
PMSF	phenylmethylsulfonyl fluoride
R_1	regulatory subunit type 1
R_2	regulatory subunit type 2
R>GC	rabbit anti-galactocerebroside
R>GFAP	rabbit anti-glial fibrillary acidic protein
RIA	radioimmunoadsorbent assay
R>MBP	rabbit anti-myelin basic protein
RNA	ribose nucleic acid
r.p.m.	revolutions per minute
RT	room temperature
SBB	standard binding buffer
SSC	standard sodium citrate
ssDNA	single stranded DNA
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
tRNA	transfer RNA

U	units
UV	ultraviolet
V/V	volume per volume
W/V	weight per volume

CHAPTER 1

HISTORICAL REVIEW

1.1. Coronavirus Overview.

The initial identification of a coronavirus (CV) came in 1937 when Baudette and Hudson isolated the agent responsible for an infectious bronchitis of chickens (Baudette and Hudson, 1933; 1937). By 1968 the similar structural features of several seemingly unrelated viral agents led to the establishment of a new viral genus termed coronaviruses (Almeida and Tyrrell, 1967; McIntosh et al., 1967; Tyrrell et al., 1968). The monogeneric family Coronaviridae, established in 1975 by the International Committee on the Taxonomy of Viruses, contains 12 species of CVs which can be grouped into 4 antigenic clusters (Wege et al., 1982; Siddell et al., 1983; Pederson et al., 1978).

Coronaviruses are responsible for a wide variety of diseases in both laboratory and domestic animals as well as in humans. Animal hosts include pigs, dogs, cats, mice, rats, cows, rabbits plus chicken and turkeys with diseases resulting from respiratory, enteric, hepatic and neurologic infection. Of considerable economic importance are infections of chickens with infectious bronchitis virus (IBV) and calves and piglets with

bovine coronavirus (BCV) and transmissible gastroenteritis virus (TGEV) respectively with resulting high mortality. Human CV infections are usually associated with upper respiratory tract infections (Larson et al., 1980; McIntosh et al., 1970) and possibly enteric infections (Caul and Egglestone 1977; Caul et al., 1975; MacNaughton and Davies, 1981).

In addition to the respiratory and enteric infections considerable interest has focused on the capabilities of murine CVs to induce a neurological disease (Weiner, 1973; Virelizier et al., 1975; Nagashima et al., 1978; Sorensen et al., 1980). The induction of a demyelinating disease in rodents following challenge with murine CV-JHMV is reminiscent of the disease process in human demyelinating diseases such as multiple sclerosis. The observation of CV-like particles in thin sections of human brain (Tanaka et al., 1976) and the isolation of two CVs from brain tissue of patients with multiple sclerosis (Burks et al., 1980) only heightened interest in these agents although these reports are as of yet unsubstantiated.

As further introduction to the studies within this thesis section 1.2 will briefly outline the general features of CV structure, replication, and assembly. The remaining sections, 1.3 and 1.4, will examine the factors influencing CV disease production and state the

objectives of this thesis respectively.

1.2. CORONAVIRUS STRUCTURE, REPLICATION, AND ASSEMBLY.

1.2.1. Coronavirus Structure and Composition.

Coronavirions appear in negatively stained preparations as large enveloped, 80-200 nm, usually spherical particles with a highly characteristic halo. The surrounding halo is comprised of widely spaced virion spikes or peplomers ranging from 12 to 24 nm in length (McIntosh, 1974). The genome is a single-stranded non-segmented RNA molecule of positive polarity ranging in mw from 5-8 X 10^6 daltons (Da) (Robb and Bond, 1979; Siddell et al., 1982). The RNA genome is capped at the 5'- end and polyadenylated at the 3'- end and is infectious when introduced into host-cells (Lomniczi, 1977; Schochetman et al., 1977).

Within the viral particle the RNA genome is found complexed with many molecules of the basic viral encoded phosphorylated nucleocapsid (NC) protein (50-60K) resulting in a long nucleocapsid structure with helical symmetry (MacNaughton et al., 1978; Stohlman and Lai, 1979). The viral envelope which encloses the nucleocapsid is derived from the intracellular membranes of the host-cell and contains two or three viral encoded

proteins E1, E2 and in some species E3 (Alonso-Caplen et al., 1984; David-Ferreira and Manaker, 1965; Massalski et al., 1982; King et al., 1985; Hogue and Brian, 1986; Callebaut and Pensaert, 1980; Dea and Tigssen, 1988).

The E1 protein (20-30K) is a glycosylated transmembrane molecule with only a small amino terminal portion exposed to the external environment (Rottier et al., 1984). The E1 protein appears to function like other matrix proteins from orthomyxo- or rhabdoviruses and may interact with the nucleocapsid structure to aid in virus particle maturation and stabilization (Holmes, 1985).

The second envelope protein, the E2 glycoprotein (180-200K) comprises the large peplomer protein extending from the envelope surface. E2 is anchored in the lipid bilayer by a short hydrophobic region, is found as either a dimer or trimer in mature peplomer form and is the only viral protein known to be transported to plasma membrane of host-cells (Cavanagh, 1983: Cheley and Anderson, 1981; Niemann and Klenk, 1981; Sturman, 1981). The E2 glycoprotein plays an important role in initiation and dissemination of virus infection being responsible for both adsorbing the virion to host-cell receptors (Holmes et al., 1981; Collins et al., 1982) and initiating cell-to-cell fusion (Sturman and Holmes, 1984).

An additional membrane protein, E3, has been found in BCV (King et al., 1985), TCV (Dea and Tijssen, 1988), human CV-OC43 (Hogue and Brian, 1986) and possibly murine CV (Taguchi et al., 1985, 1986). This protein is poorly characterized at this stage but appears to be a glycoprotein of approximately 65 KDa and may be responsible for the hemagglutinating capacity of BCV, TCV and OC43. The protein may also function as a receptor-destroying enzyme since recent studies have associated acetylesterase activity with this protein (Vlasak et al., 1988) analagous to previous reports with the HE glycoprotein of influenza C virus (Vlasak et al., 1987).

Mature CV particles do not contain an RNA-dependent RNA polymerase and thus must synthesize it upon infecting host-cells. In addition, there has been a report of a virion associated protein kinase (Siddell et al., 1981) but of what function and whether of host or viral origin remains to be answered.

1.2.2. Coronavirus Replication.

Following CV adsorption to host-cell receptors, viral particles are believed to penetrate mainly via receptor mediated endocytosis into endosome fractions although direct fusion of viral and cellular membranes may also occur (Krzystyniak and Dupuy, 1984; Mizzen et

al., 1985; Coulter-Mackie et al., 1985). Evidence employing lysomotropic agents, such as chloroquine and ammonium chloride, have indicated that during the uncoating stage a low-pH dependent fusion between viral and endosome membranes is required for release of nucleocapsid structure into cytoplasmic environment (Mallucce, 1966; Mizzen et al., 1985; Coulter-Mackie et al., 1985). Upon release of nucleocapsid the parental viral genome undergoes primary translation of at least the 5' end which probably encodes the RNA-dependent RNA polymerase necessary for initiation of virus replication (Brayton et al., 1982). The viral RNA polymerase then transcribes a full-length complementary negative-strand RNA which contains a poly-U tract at the 5'-end (Lai et al., 1982; Sawicki and Sawicki, 1986). This negative-stranded full-length RNA then serves as a template for the synthesis of full-length positive sense genomic RNA as well as for the synthesis of 6-7 sub-genomic mRNA through a discontinuous transcription process (Spaan et al., 1981; Cheley et al., 1981). The mRNA are 5'-capped and 3'-polyadenylated and form an overlapping set of 3'-co-terminal messages with mw's ranging from 0.6-3.7 X 10^6 Da (Spaan et al., 1981; Cheley et al., 1981). The viral mRNAs, produced in non-equimolar ratios which remain constant throughout an infection (Stern and Kennedy, 1980; Leibowitz et al., 1981), have only the

5'- end of each message translated so that each mRNA is functionally monocistronic (Siddell et al., 1982). Coronaviruses have developed a unique approach to allow for independent synthesis of mRNA's employing a common leader sequence of about 70-72 nucleotides which acts as a primer for RNA polymerase binding at internal sites on the negative stranded template (Baric et al., 1983; Brown et al., 1984; Lai et al., 1984; Spaan et al., 1983). The leader is independently synthesized from the 3'-end of the negative strand template and then binds to highly conserved intergenic junction sequences to act as primer for mRNA synthesis (Spaan et al., 1982, 1983; Lai et al., 1984).

Based on the evidence of leader-primed transcription two models have been proposed to account for the non-equimolar production of viral mRNAs within host-cells. One possible model involves the free leader segment binding to the conserved intergenic regions within the negative-strand template with differing affinities. The leader binding would be based upon the degree of complementarity between the leader and the intergenic sequence (Spaan et al., 1983; Budzilowicz et al., 1985; Shieh et al., 1987). Based on this model, the most abundant mRNA would have a corresponding intergenic region with the highest complementarity to

the free leader sequence.

More recently a second possible model was proposed which explains the differences in mRNA levels as being controlled by differential premature termination of transcription (Konings et al., 1988). In this model, initation of mRNA transcription is believed to occur as before with binding of free leader to intergenic sequences. However, relatively slow new initiation events at the intergenic regions are believed to in effect block the elongation of previously initiated transcription complexes. This results in a temporary pausing of the transcription complex with resulting premature termination of transcription. The premature termination of transcription will affect longer transcripts to a greater extent which results in the non-equimolar gradient of viral mRNAs. Further studies will be required to fully understand this complex sequence of events regulating CV replication.

1.2.3. Synthesis of Viral Proteins.

Synthesis of viral proteins is not dependent on host-cell nuclear functions (Brayton et al., 1981) and occurs independently within the cytosol on host polysomes (Cheley and Anderson, 1981; Leibowitz et al., 1982). While all viral proteins are produced within the cytosol there are spatial differences in sites of

synthesis. The synthesis of NC protein as well as several non-structural proteins occurs on free polysomes while the glycoproteins E1 and E2 are translated on polysomes associated with the rough endoplasmic reticulum (Leibowitz et al., 1982; Niemann and Klenk, 1981).

The NC protein is the most abundantly produced protein within infected cells and is rapidly phosphorylated on serine residues (Siddell et al., 1981; Cheley and Anderson, 1981). The NC protein binds to new genomic RNA to form nucleocapsid structures before being packaged as mature virions. NC protein may also be involved in additional functions since antibodies against NC inhibit in vitro genomic RNA synthesis (Compton et al., 1987).

Several of the non-structural proteins found within infected cells or their corresponding in vitro translation products have been characterized although their function remains an enigma (Dennison and Perlman, 1987; Soe et al., 1987; Ebner et al., 1988; Leibowitz et al., 1988; Smith et al., 1987).

The membrane proteins E1 and E2, while spatially localized, have differences in post-translational processing (Holmes et al., 1981). The E1 glycoprotein differs from E2 in being glycosylated on serine or threonine residues and not being transported to the

plasma membrane (Holmes et al., 1981; Neiman et al., 1982). In contrast the E2 glycoprotein is glycosylated on asparagine residues (Cheley and Anderson, 1981) is acylated and oligosaccharide modified in the golgi apparatus (Schmidt, 1982; Stern and Seffton, 1982) and may be proteolytically cleaved within the golgi or at the plasma membrane (Frana et al., 1985).

1.2.4. Virion Assembly.

Ultrastructural analysis of CV infected cells has revealed mature coronavirions budding into the cisternae of the golgi and endoplasmic reticulum apparatus (David-Ferreira and Manaker, 1965; Massalski et al., 1981). The precise sequence of events remains to be elucidated but probably involves the interaction of nucleocapsid structures with those organelle membranes containing the E1 and E2 glycoproteins (Massalski et al., 1982). It is important to note however that E2 may not be necessary for virion maturation since viral particles are formed in tunicamycin treated cells (Holmes et al., 1981). Mature viral particles may be released from infected cells either by migrating through the secretory apparatus via smooth walled vesicles to the plasma membrane (Doughri et al., 1976; Toose et al., 1984, 1987) or by cell lysis (Chasey and Alexander, 1976).

1.3. FACTORS INFLUENCING CORONAVIRUS PATHOGENESIS.

1.3.1. Overview.

Coronaviruses and in particular animal CV, infect a wide range of organs resulting in a correspondingly wide range of diseases. The best characterized infections involving animals have indicated complex interactions between virus and host in establishing the pathogenetic potential of the virus. A wide range of variables including route of virus inoculation (Hirano et al., 1975, 1981), genetic background of animal (Levy-Leblond et al., 1979; Stohlman and Frelinger, 1978; Wilson and Dales, 1988), virus isolate and dose (Hirano et al., 1980; Sorensen et al., 1980), immune status of animal (Sorensen et al., 1982; Wege et al., 1983), age of animal at time of virus challenge (Sorensen et al., 1980, 1982; Wege et al., 1984), and in particular reference to the nervous system, the state of host-cell differentiation (Beushausen and Dales, 1985; Wilson et al., 1986) have been implicated in determining the outcome of CV challenge. This section of the review will examine some of these points in more detail especially as these factors relate to control of murine CV-induced disease in the nervous system of rodents which is the main topic of this thesis.

1.3.2. Route of Virus Inoculation, Serotype, and Dose.

The type of disease which develops following virus challenge depends on the route of inoculation, virus serotype, and dose used (Hirano et al., 1981, 1975; Weiner, 1973). The JHMV strain, considered the most neurotropic, causes neurological disease following intraperitoneal (ip), intranasal (in), and intracranial (ic) injections and only weakly involves other organs when given in large doses in conjunction with immunosuppression. A59, MHV_3 and MHV_2 are more viscerotropic usually causing acute hepatitis following ip or iv inoculation although all three are capable of infecting the nervous system when introduced ic. Several other strains of MHV such as MHV-S and MHV_1 demonstrate low pathogenicity by any route of administration with diseases such as hepatitis manifesting only after giving large doses (10^6 pfu) of virus in immunosuppressed hosts.

The complex interactions between virus serotype and host in determining disease potential will become more evident in the next section concerning the effects of host-age and genotype on CV pathogenicity.

1.3.3. Host-Age and Genotype.

The type and severity of disease produced following virus challenge is dependent upon both virus and host

factors. Perhaps the best method for examining the interplay of these parameters is to look at a few examples in more detail.

Early work by Bang and Warwick (1960) with ip inoculations of adult mice with MHV_3 established a genetically based host resistance to acute hepatic disease. Thus, highly resistant strains of mice, such as the A/J strain, survive challenge with even high doses of MHV_3 while highly susceptible strains such as C57BL/6 succumb to acute hepatitis soon after infection (Le Provost et al., 1975). F1 hybrids between resistant and susceptible mice produce offspring described as semi-resistant with only a small fraction of infected animals showing acute disease production (Le Provost et al., 1975; Virelizier et al., 1975). The responses of these resistant mice is not, however, completely independent of age. A/J mice while still much more resistant to MHV_3 challenge than susceptible controls at 6 weeks of age, do not express complete resistance until they are 10-12 weeks old. The genetic basis for this resistance has been reported to be controlled by at least 2 genes, 1 of which is recessive and controls susceptibility (Levi-Leblond et al., 1979). This genetically controlled resistance to liver disease appears to be expressed at the level of the macrophage since isolated in vitro primary cultures of macrophages

reflect the control of disease observed in vivo (Sabesin and Koff, 1974; Virelizier and Allison, 1976).

This example illustrates the relatively simple association of host-genotype resistance with a single MHV serotype. A somewhat more complicated situation occurs however when we compare MHV_3 and JHMV infections in the nervous system of rodents.

SJL mice exhibit an age and virus sertoype related resistance to CV induced neurological disease following ic challenge (Stohlman and Frelinger, 1978; Knobler et al., 1981; Wilson and Dales, unpublished observations). Twelve week old SJL mice demonstrate complete resistance to JHMV following ic challenge but remain susceptible to the highly related strain MHV_3. Genetic studies of this resistance are divided with reports suggesting either a single locus (Knobler et al., 1981) or 2 non-H2 linked loci (Stohlman and Frelinger, 1978) controllng the infection. The in vivo resistance of SJL mice to JHMV has also been correlated with in vitro primary cultures of neurons (Knobler et al., 1981), astrocytes (Collins et al., 1983), and macrophages (Knobler et al., 1984; Stohlman and Frelinger, 1981) from neonatal mice. This subject will be discussed in greater detail in Chapters 4 and 5 where results concerning the possible mechanism for this resistance are presented.

One further example outlining the interaction

between virus serotype and host in determining disease production concerns the differing responses of inbred strains of rats to MHV-JHMV and MHV_3. In-vivo ic challenge of adult or weanling Wistar Furth (WF) rats with JHMV leads to neurological disease of either acute or chronic nature (Nagashima et al., 1978; Sorensen et al., 1980). Similar challenge with MHV_3 fails to induce any detectable neuropathology (Hirano et al., 1980; Sorensen et al., 1980). This is in contrast to ic challenge of mice where both MHV_3 and JHMV are capable of eliciting neurological disease (Le Provost et al., 1975; Sorensen et al., 1982). These findings have been confirmed through in vitro studies employing primary cultures of oligodendrocytes and astrocytes established from the neonatal cortices of both rats and mice (Beushausen and Dales, 1985; Wilson et al., 1986). When primary cultures of glial cells from rat are challenged, JHMV shows an unambiguous tropism for oligodendrocytes and MHV_3 for astrocytes (Beusha[illegible]n and Dales, 1985). Similar infections of primary mouse cultures fail to discriminate between the two viruses so that oligodendrocytes and astrocytes replicate both MHV_3 and JHMV (Wilson et al., 1986). Thus there appears to be a correlation between the capabilities of CVs to infect oligodendrocytes in vitro and the ability of these viruses to cause a neurological disease following ic

challenge.

One further parameter controlling CV replication in particular reference to the nervous system stems from the observations of Sorensen et al., (1980; 1982) who demonstrated an age-related insusceptiblity to ic challenge with JHMV in WF rats. This age-related resistance to JHMV occurring about 21 days after birth suggested a differential host-cell control over CV infection, possibly related to the state of nervous system maturation. When in vitro primary cultures of oligodendrocytes from both rats and mice were induced to differentiate with the metabolite dibutyryl cAMP, the cultures proved to be resistant to JHMV infection (Beushausen and Dales, 1985; Wilson et al., 1986). It appears that the state of oligodendrocyte differentiation determines the susceptibility of that cell-type to CV infection. This topic will be discussed in greater detail in Chapters 2 and 3 where results are presented outlining a possible mechanism for this observed resistance.

Any discussion of viral-induced pathology requires an examination of the immune status of the host. Both cellular and humoral immunity are important factors in determining the course of CV induced pathology and will be discussed in the next section.

1.3.4. Immune Status of Host.

Cellular immunity, involving both natural killer (NK) cells (Bukowsky et al., 1983) and T-lymphocytes (Kai et al., 1981; Stohlman et al., 1986; Yamahuchi et al., 1988), have been implicated in clearing virus from infected hosts. Researchers have reported the induction of both Major Histocompatibility (MHC) Class I (Suzumura et al., 1986; Lavi et al., 1987) and Class II (Massa et al., 1987) antigens on the surface of glial cells following MHV infection, allowing clearance of virus infected cells within the CNS by cytotoxic T-cells. Humoral immunity is also important in CV infections since both polyclonal and monoclonal antibodies, especially to the E2 glycoprotein, have been shown to modulate MHV infection (Buchmeier et al., 1984; Talbot et al., 1985a, 1985b; Pickel et al., 1985; Perlman et al., 1986).

These examples serve to illustrate the complexity of responses, involving both virus and host, controlling CV pathogenicity. Susceptibility to neurological disease following challenge with CV is not only highly dependent of strain of virus used but also on host factors including age of animal at time of challenge, genetic components and immune status.

1.4. OBJECTIVES.

The primary objective of this thesis was to examine the possible mechanisms relating to host-cell control over CV replication within the nervous system of rodents. Emphasis was placed on characterizing the specific cell-virus interactions occurring within glial cells from rodent CNS.

In an effort to simplify these studies, in vitro primary cultures of glial cells containing oligodendrocytes and astrocytes from both rats and mice were employed. These cultures, both mixed and isolated, have previously been shown to mimic their in vivo counterparts in both temporal (Abner et al., 1981) and morphologic characteristics (McCarthy and deVellis, 1980).

Specifically, Chapters 2 and 3 will examine the possible mechanism of age related insusceptibility to JHMV associated with nervous system maturation in rats. Chapters 4 and 5 present findings relating to the resistance of SJL mice to JHMV. The last chapter, Chapter 6, summarizes the possible mechanisms of host-cell resistance presented within these studies.

CHAPTER 2

CHARACTERIZATION OF CORONAVIRUS INFECTION IN PRIMARY EXPLANTS OF MOUSE CNS.

2.1. INTRODUCTION.

In studying the factors involved in the development of demyelinating diseases it has been demonstrated that several strains of murine coronaviruses (CVs) including MHV-A59 (Lavi et al., 1984), MHV_3 (Hirano et al., 1981; Sorensen et al., 1982), and JHMV (Cheever et al., 1949; Pappenheimer, 1958; Weiner, 1973; Stohlman and Weiner, 1981; Hirano et al., 1981; Sorensen et al, 1982) can cause neurological disease in rodents.

Previous findings using mouse central nervous system (CNS) cultures demonstrated that both neurons and astrocytes can be infected with JHMV (Knobler et al., 1981b; Dubois-Dalcq et al., 1982; Collins et al., 1983). Concerning oligodendrocytes of rodents, which have been implicated as the in vivo targets, those from the rat are indeed infectable in vitro by JHMV (Beushausen and Dales, 1985). Comparable in vitro information about murine oligodendrocytes is, however, lacking (Knobler et al., 1981a, 1981b; Dubois-Dalcq et al., 1982; Collins et al., 1983).

Evidence at hand suggests that there are fundamental species differences in regulation of the

infectious process, whereby mouse neural cells can be infected by several CVs indiscriminately (Hirano et al., 1981; Sorensen et al., 1982; Lavi et al., 1984), but oligodendrocytes and astrocytes explanted from rat brain discriminate unambiguously between JHMV and MHV_3 (Beushausen and Dales, 1985). Thus, the in vitro establishment of dispersed primary murine glial cultures, including oligodendrocytes, would enable a direct comparison of the interaction between different CVs with the cells from the CNS of the two species. Furthermore, since in such in vitro primary cultures a "time clock" of development follows the same sequence as that which occurs in vivo (McCarthy and De Vellis, 1980; Abney et al., 1981; Barbarese and Pfeiffer, 1981; Bhat et al., 1981; Bologa-Sondru et al., 1981), such cultures may be used to better define the virus-glial cell interactions involved in the development of disease in the CNS.

2.2. MATERIALS AND METHODS.

2.2.1. Continuous Line of Cells.

L-2 mouse fibroblasts (Rothfels et al., 1959) were routinely propagated in suspension at 37 degrees Celcius (C) on rotary shaker (140 rpm; New Brunswick Psychotherm) in Eagle's MEM (GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 ug/ml streptomycin, plus 0.1% methylcellulose. Cells were also grown as monolayers at 37 C in humidified atmosphere plus 5% CO_2 in MEM minus methylcellulose.

2.2.2. Viruses.

The MHV_3 and JHMV strains of the mouse hepatitis virus (MHV) were propagated and plaque assayed on L-2 mouse fibroblasts as follows: subconfluent cultures of L-2 cells were absorbed for 1 hr at room temperature with virus at a multiplicity of infection (MOI) of 1 plaque forming unit (PFU) per cell. Cultures were then shifted to 37 C until 50% of cells had lifted off. The remainder of the monolayer was scraped off using a rubber policeman and passed through a syringe fitted with a 21-gauge needle. The resulting lysate was sedimented at 2000 x g for 10 min to remove cellular debris and the supernatant further centrifuged at 70,000 x g for 60 min to pellet the virus. The pelleted virus was resuspended in a small volume of MEM plus 10% FBS,

aliquoted, and stored at -70 C. Infectious virus was quantitated by plaque assays, as previously described by Lucas et al., (1977) and outlined in the next section. Virus stocks were prepared from twice plaque purified isolates available within our laboratory.

The Indiana strain of vesicular stomatitis virus (VSV) was propagated by infecting L-2 cell monolayers, MOI 1.0, and incubating the cells 37 C for 24 hrs. The supernatant, containing released progeny virions, was then harvested and cellular debris removed by low speed centrifugation as outlined above. The resulting suspension was then passed through a 0.22 um Millex-GS filter (Millipore), aliquoted and stored -70 C.

2.2.3. Virus Titration.

To quantify release of extracellular virus or virus stocks the plaque assay technique of Lucas et al., (1978) was used. Monolayers of L-2 cells in 35 mm tissue culture plates (Gibco) were adsorbed for 1 hr at room temperature (22 C) with 0.2 ml of virus inoculum on a rocking platform. Unadsorbed inoculum was removed, and plates washed with 2 mls MEM before incubating cultures at 37 C for 24 hr with 2 ml of MEM containing 0.5 % methylcellulose (4000 cps). Monolayers were subsequently fixed for 20 min with 10% formaldehyde, supernatant removed, and cultures stained 10 min with 0.1% crystal violet. Plates were then washed in running H_2O,

air dried and plaques scored. Virus titrations were usually reported as plaque forming units (PFU) per ml.

2.2.4. Primary Murine Glial Cultures.

Primary glial cultures were prepared with slight modifications as described by Beushausen and Dales (1985). Briefly, twenty 1- to 2- day-old mouse pups were decapitated, the meninges dissected away, and the cerebral hemispheres removed and placed in ice-cold BME_{10} consisting of basal minimum essential medium (BME) (Gibco), supplemented with 10% heat inactivated FBS, sodium bicarbonate (1 g/liter), 0.6% dextrose, and gentamycin (10 ug/ml, Schering Pharmaceuticals). The isolated hemispheres were washed three times with 10 ml of cold BME_{10} to displace any leftover meninges and contaminating red blood cells (RBC), then were triturated through a 10-ml pipet thereby producing a homogenous suspension of free cells. The larger aggregates and debris were removed by filtration through a Nitex 130-um mesh and the filtrate containing monodispersed cells centrifuged for 5 min at 40 x g to pellet the cells. The cell pellets derived from 40 hemispheres were resuspended in 10 volumes of BME_{10}, plated into 175-cm^2 culture flasks (Nunc), and maintained at 37 C in a humidified atmosphere containing 5% CO_2 for 4 days before changing medium.

To obtain cultures enriched in either oligodendrocytes or astrocytes, minor modifications to

those of Beushausen and Dales (1985), were made, based on the techniques of McCarthy and de Vellis (1980), as adapted by McMorris (1983). These primary cultures were propagated for approximately 2 weeks, with medium changes every 2-3 days, and used to obtain oligodendrocyte-enriched cultures as follows. The BME_{10} medium was replaced with 30 ml of warm phosphate-buffered saline (PBS), pH 7.4. The flasks were then shaken vigorously by hand to release the less adherent oligodendrocytes, the amount of cell release being monitored under phase-contrast optics. The free cells were centrifuged at 40 x g for 5 min and cell pellets resuspended in BME_{10}, the volume being adjusted to give the desired cell concentration, usually about 3×10^5 cells/ml. After dispensing into 24-well plates the cells were allowed to become attached for 24 hr at 37 C before use.

Cultures enriched in astrocytes were produced using tightly adherent cells remaining in flasks from which oligodendrocytes had been removed. The adherent cells were released with 0.25% trypsin in PBS, and the cell suspension diluted with BME_{10} was then centrifuged at 40 x g for 5 min. Cell pellets were resuspended in a volume of BME_{10} adjusted to give a suspension of 3×10^5 cells/ml and the astrocytes dispensed into 24-well plates. Cells used for immunofluorescence were grown on 12-mm glass coverslips (Chance, Propper Ltd.). Mouse

strains CD.1 and DBA/2 were purchased from Jackson Laboratory, Bar Harbor, Maine.

2.2.5. Infection Conditions For Primary Cultures.

Cultures in 24-well plates were absorbed with 0.2 ml virus suspension at a MOI of 1 at 37 C for 1 hr. Following adsorption virus was removed and cultures washed three times with 1 ml BME_{10} and then incubated with 1 ml per well of medium at 37 C.

2.2.6. Preparation of Antisera.

The techniques used for the production of antisera and the immune-labeling of cells have been previously reported (Beushausen and Dales, 1985). Briefly, antibodies specific against (>) galactosyl cerebroside (>GC) were prepared from rabbit antiserum by passing the serum sequentially through columns of protein A-Sepharose, to select out the IgG, then through BSA-Sepharose to remove contaminating BSA antibodies. The avidity of >GC IgG was tested by radioimmunnoassay (RIA) of Holmgren et al., (1980), as adapted for use with GC by Raine et al., (1981). Rabbit polyclonal sera to bovine glial fibrillary acidic protein (GFAP) was purchased from Cedarlane, Labs Ltd. Rabbit polyclonal sera to myelin basic protein (MBP) was obtained through the courtesy of Dr. Zobeeda Hossein. Monoclonal antibodies (Mab) against JHMV nucleocapsid antigen were kindly supplied by Dr. M. Buchmeier, Scripps Clinic and

Research Foundation, La Jolla, California.

2.2.7. Immune Labeling of Cells.

Cultures of glial cells on glass coverslips were washed briefly in PBS (pH 7.2), and then fixed for immunofluorescence by immersion in acetone for 2 min at 22 C (Manthorpe et al., 1979). Following fixation coverslips were washed for 20 min with 4 changes of PBS in 50 ml beaker before proceeding to immune-labeling as described in Dales and Oldstone (1982). Cultures were sequentially reacted with 50 ul volume of specific antibodies for 30 min at 22 C followed by fluorochrome-labeled antibody conjugates to mouse or rabbit immunoglobulin for an additional 30 min at 22 C. Cultures were washed five times with 5 mls PBS in between and after labeling of cells with specific antibody and fluorochrome conjugates. Cultures were then mounted on glass slides with a 10% glycerol in PBS solution and viewed under UV optics with a Wild-Leitz, Dialux 20 microscope. The final dilutions of specific antibodies were as follows: R > GC, 1:1; R> MBP, 1:9; R > GFAP, 1:39. Fluorochrome conjugates G>R-Rhodamine and G > mouse fluorescein were both used at 1:19 dilutions. Polyclonal goat > mouse fluorescein (G > mu FITC), goat > rabbit rhodamine (G > ra Rho) and rabbit > bovine rhodamine (Ra > bo Rho) conjugates were purchased from Cappel Laboratories, Inc.

2.2.8. Immune Lysis of Infected Cultures.

Primary cultures of oligodendrocytes or astrocytes were infected with JHMV, assayed by virus titration 24 hr post-inoculation and then subjected to immune lysis using >GC antibodies and rabbit Lo-Tox complement (Cederlane, lab Ltd.), as previously described (Beushausen and Dales, 1985) and outlined below.

Infected glial cultures were washed twice with 1 ml cold PBS, pre-cooled at 4 C for 10 min in BME without serum, and then incubated for 60 min at 4 C with > GC antibodies (Ab) diluted 1:2 in BME without serum. Following Ab labeling cells were washed three times with cold PBS and then incubated at 37 C in humidified atmosphere with rabbit Lo-Tox complement diluted 1:15 with BME. Cultures were washed twice with 1 ml BME to remove complement and then were incubated at 37 C with 2 mls BME_{10}. Virus titrations were assayed 24 hr post-treatment for PFU/ml of culture supernatant.

2.2.9. Preparation of Glial Cell Extracts.

Cultures of mixed glial cells from DBA/2 mice in 24 well tissue cultures dishes (Nunc) were used for assaying induction of 2':3'-cyclic nucleotide 3'-phosphohydrolase (CNPase), following N6,2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate (dbcAMP, Sigma) treatment. Following treatment glial cells were harvested by rinsing monolayers twice with 2 mls of

0.15M NaCl and cells were scraped off using teflon policemen. Cells were pelleted by centrifugation at 150 x g for 2 min at 4 C. Pellets were resuspended in 500 ul of deionized water containing 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and a 300 ul aliquot of this suspension was mixed with 2 volumes of 1% sodium deoxycholate in H_2O. Samples were incubated 10 min on ice and then homogenized with 20 strokes in 1 ml Potter-Elvehjem homogenizer. Cell extracts were aliquotted and stored -70 C. Protein determinations were done according to Lowry et al., (1951) using bovine serum albumin (BSA) as the standard.

2.2.10. Treatment of Primary Cultures With Dibutyryl Cyclic AMP and Assay of 2':3' Cyclic Nucleotide-3'-Phosphohydrolase.

Cultures of glial cells in 24-well plates were incubated with 1 ml BME_{10} in the absence or presence of dbcAMP, at a final concentration of 1 mM. Cultures were maintained at 37 C and medium changed every 2 days for duration of the study. The methods used for determination of the enzyme CNPase were those developed by Prohaska et al., (1973), as modified by McMorris (1983).

Briefly, an aliquot of glial cell extract (20 ug protein) in 100 ul volume was mixed with 100 ul of 50 mM Tris-HCl (pH 6.2), containing 15 mM 2':3' cAMP (Sigma)

and incubated for 10 min at 30 C. CNPase reaction was terminated by boiling the mixture for 1 min followed by quenching on ice. Reaction mixtures were then incubated for 30 min at 30 C following addition of 100 ul of 100 mM glycine buffer (pH 10.2) containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 2.5 U calf intestine alkaline phosphatase (Boehringer-Mannheim, 2500 U/mg). Following this incubation the free inorganic phosphate was extracted by adding 1 ml of 1:1 isobutanol:benzene and 1 ml of 1.5 % ammonium molybdate (Aldrich) in 0.5 N H_2SO_4. The resulting mixture was vortexed for 20 sec followed by centrifugation at 650 x g for 5 min. The upper phase was removed and absorbance determined in spectrophotometer at 410 nm using isobutanol-benzene as blank. Under these conditions one unit of enzyme activity was defined as that amount which produces 1 umole of 2'AMP from 2':3'cAMP/min.

2.3. RESULTS.

2.3.1. Characterization of the Cell Types in Primary Explants from Mouse Brain.

Primary glial cultures, termed mixed cultures, examined under phase-contrast optics contained predominantly two morphological cell types, organized in stratified layers. The bottom layer was occupied by large, flat, tightly adherent cells typical of astrocytes (McCarthy and de Vellis, 1980). The upper layer contained more widely dispersed cells possessing smaller cell bodies of greater phase density and extensive processes, morphologically like oligodendrocytes of the rat (McCarthy and de Vellis, 1980; Pfeiffer et al., 1981; Beushausen and Dales, 1985). Due to the differential adhesiveness of these cell types the less adherent cells, at the surface, could be removed by manual shaking so as to provide relatively pure oligodendrocytic cultures (Fig. 2.1). Also present occasionally in low number, within the mixed cultures, were cells with large cell bodies and asymmetric processes, usually consisting of a single long process on one side and several smaller extensions on the opposite side of the cell body, reminiscent of neuronal morphology.

More definitive identification of the cell types involved was undertaken by means of indirect

Figure 2.1. Culture enriched in oligodendrocytes of CD.1 mice, viewed under phase-contrast 24 hr after plating, following release from a mixed culture. x 600.

immunofluorescence, using antibodies to cell-type specific marker antigens. Antibodies to MBP were used for identifying oligodendrocytes and to GFAP for astrocytes. Judging by the fraction of MBP positive cells, between 80% and 90% of cells present in enriched cultures were oligodendrocytes. In the astrocytic cultures over 99% of cells were GFAP positive.

2.3.2. Identification of Cell Types Expressing Viral Antigen.

With glial cells from the rat there is a CV strain-related specificity, whereby oligodendrocytes support the replication of JHMV and astrocytes of MHV_3 (Beushausen and Dales, 1985). Published studies with murine CNS cultures (Dubois-Dalcq et al., 1982) however suggest that exclusive tropisms of this type may not prevail in the mouse. To test this idea using a better defined system, we examined replication of JHMV and MHV_3 in dispersed cultures from mouse brain, consisting predominantly of either oligodendrocytes or astrocytes. For this purpose the cells were grown on glass coverslips and infected with either JHMV or MHV_3 at a MOI of 1. The infection was allowed to proceed at 37 C for 30 hr before processing for immunofluorescence.

In the case of oligodendrocytic cultures, dual indirect tagging using Rho and FITC with Mab against JHMV nucleocapsid antigen as well as polyclonal

antibodies to MBP, revealed that oligodendrocytes became infected with both JHMV and MHV_3 (Figs. 2.2 A-F). Likewise dual labeling with >GFAP antibodies and > nucleocapsid Mab showed that astrocytes were also infectable with JHMV (Figs. 2.3 A,B) and MHV_3 (data not shown).

To better quantify the percentage of cells that are positive for virus antigen, independent cultures of oligodendrocytes and astrocytes from CD.1 mouse brain were inoculated at a MOI of 1 PFU/cell and monitored on the 1st and 3rd days by immunofluorescence for infected cells as outlined above. The results, summarized in Table 2.1, show that by Day 1 the percentage of oligodendrocytes infected with JHMV was 52, and with MHV_3, 65. By the 3rd day the frequency of infection for both viruses increased to approximately 82%. Similar results were obtained with astrocytes, although the percentage of infected cells was less. Virus-positive astrocytes ranged from 20 to 33% on the 1st day and from 22 to 42% on the 3rd day for MHV_3 and JHMV, respectively. Thus it appears that spread of infection and cell killing occur somewhat more rapidly with oligodendrocytes than with astrocytes.

2.3.3. Species and Glial Cell Type Related Differences in Coronavirus Replication.

Quantitative data concerning virus production

Figure 2.2. Cultures, as in Figure 2.1, were infected with coronaviruses for 30 hr prior to fixation and staining. A-C infected with JHMV; D-F infected with MHV_3. A, D viewed by phase-contrast; B, C, E, F examined under U.V. illumination. B, E reacted with Mab>JHMV nucleocapsid and G>mu FITC; C, F reacted with polyclonal >MBP antiserum and G>ra Rho. Note the extensive process formation of the oligodendrocytes. In B, C arrows point towards oligodendrocytes formed into a syncytium following infection. x1700.

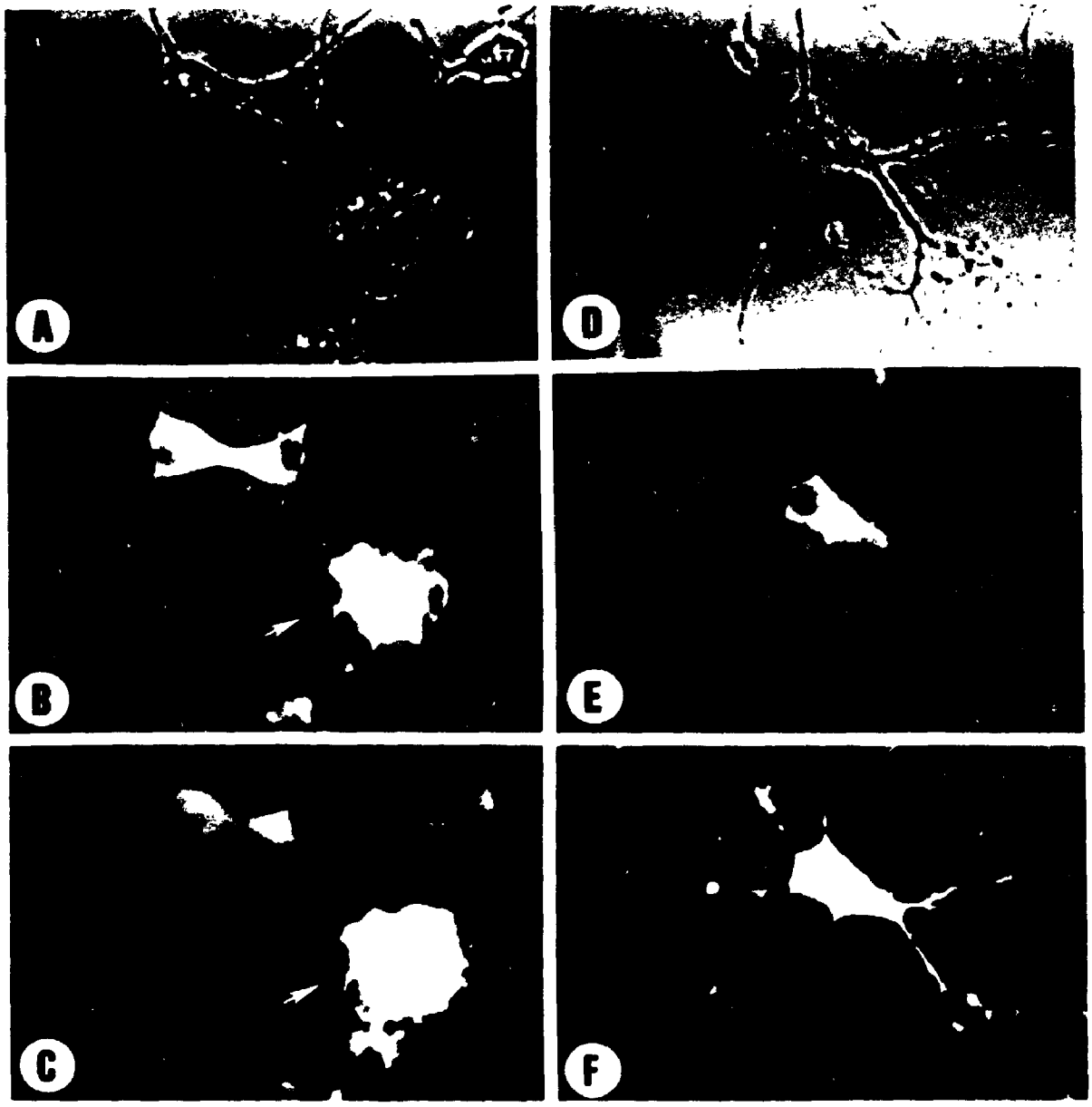

A
D
B
E
C
F

Figure 2.3. The appearance under U.V. optics of an astrocyte culture from CD.1 mice, fixed 54 hr after plating and 30 hr following infection with JHMV. In A reacted with polyclonal >GFAP antiserum and Ra>bo Rho. Note the massive bundles of filaments containing GFAP antigen (arrowheads). In B reacted with Mab>JHMV nucleocapsid antigen and G>mu FITC. Multinucleated cells are identified by arrows. x 1600.


B

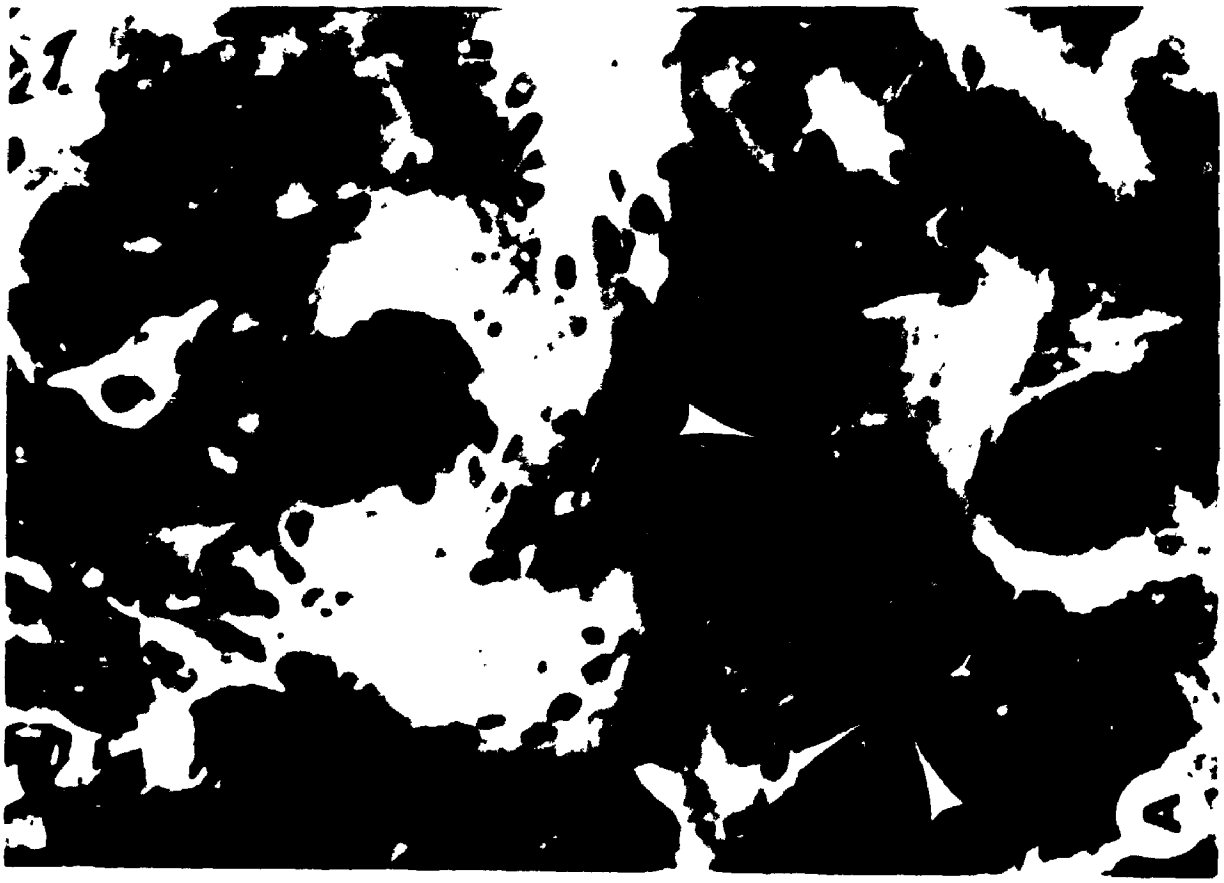

A

Table 2.1

Frequency of Coronavirus Antigen-Positive Cells Within Glial Cultures Determined by Immunofluorescence:

Virus Strain	Days after inoculation	Nuclei per Syncytium (Average and standard deviation)	Percentage of positive cells and standard deviation
		Oligodendrocytes	
JHMV	1	4.8 ± 1.02	52.0 ± 4.93
	3	8.2 ± 1.96	82.4 ± 7.83
MHV_3	1	5.7 ± 0.97	65.4 ± 7.41
	3	6.6 ± 1.25	82.8 ± 8.04
		Astrocytes	
JHMV	1	5.6 ± 3.37	32.8 ± 3.66
	3	5.4 ± 1.80	41.6 ± 11.67
MHV_3	1	6.0 ± 1.69	20.0 ± 4.56
	3	6.6 ± 1.80	22.4 ± 5.01

The total number of nuclei counted in random fields of oligodendrocytes ranged from 783 to 1245. Similarly, in astrocyte cultures the total number of nuclei ranged from 477 to 632.

complementary to observations from immunofluorescence were obtained by infecting murine cultures enriched in oligodendrocytes or astrocytes and assaying supernatant medium for amount of PFU released. The results obtained, summarized in Table 2.2, showed that both JHMV and MHV_3 could replicate with about equal facility in oligodendrocytes and astrocytes explanted from brains of CD.1 mice. To establish this fact care had to be exercised to ensure that the cells employed were at approximately equal, uniform density. The profound decrease in yields of PFU from these glial cultures, between the 1st and 3rd days was almost certainly the consequence of cytopathic effects, which became evident within 48 hr after infection.

Glial cells from rat brain were, by contrast, discriminating hosts for replication of JHMV and MHV_3, as evident in Table 2.2, confirming previous findings that JHMV was tropic for oligodendrocytes and MHV_3 for astrocytes of this species (Beushausen and Dales, 1985). Therefore, a clear-cut species difference in control of CV replication has been demonstrated.

2.3.4. Immune Lysis of Oligodendrocytes.

To further determine the uniformity of oligodendrocytic cultures, cells producing CV were subjected to complement-mediated immune lysis and tested for yields of PFU. It is evident from the data in Table

Table 2.2

Species Related Differences in the Replication of Coronaviruses in Glial Cultures:

Cultures from	Days after inoculation	Titer x 10^2 PFU/ml[a]			
		Oligodendrocytes[b]		Astrocytes[b]	
		JHMV	MHV_3	JHMV	MHV_3
CD.1 mice	1	4,500(8)	7,000(6)	3,900(8)	6,200(6)
	3	1,400(6)	190(2)	550(4)	260(4)
Wistar Furth rats	1	12(4)	0(4)	0(4)	34(2)
	3	20(4)	0(4)	0(4)	15(2)

a) the values are averages from the number of experiments shown in parentheses.

b) cell density was approximately 3 x 10^5 cells/well.

2.3 that only cultures enriched for oligodendrocytes almost ceased producing JHMV after exposure to both GC antibodies and complement, but were essentially unaffected by either >GC antibodies or complement when added separately. Virus production by astrocyte cultures was unaffected by addition of combined GC antibodies and complement (Table 2.3).

2.3.5. Relationship between Induction of Oligodendrocyte-Associated Enzyme 2':3'-Cyclic Nucleotide-3'-Phosphohydrolase (CNPase) and Virus Replication.

CNPase activity is associated with myelin-forming cells of the nervous system. In rat oligodendrocytes treated with dbcAMP the specific activity of this enzyme is greatly enhanced (McMorris, 1983), while replication of JHMV is arrested (Beushausen and Dales, 1985). This inducer, however, has limited influence on production of MHV_3 in rat astrocytes following treatment with the drug for 2 days. Since murine oligodendrocytes support the replication of both JHMV and MHV_3, it was necessary to determine whether, upon induction of differentiation, these cells ceased to replicate both coronaviruses.

The time-related induction of CNPase activity was examined using mixed primary glial cultures treated continually with 1 mM dbcAMP. Enzyme was assayed in duplicate samples of controls and treated cells at

Table 2.3

Effect Of Immune Lysis On The Replication Of JHMV In CD.1 Mouse Astrocyte And Oligodendrocyte Cultures:

Treatment of Culture	Virus produced x 10^2 PFU/ml			
	Astrocytes[a]		Oligodendrocytes[a]	
	before treatment[b]	after treatment	before treatment	after treatment
anti-GC Ab	780	7400[c]	7.5	4
complement	790	5600	16	15
anti-GC Ab + Complement	1300	5900	10	0.5

a) cell density was approximately 1 x 10^5 cells/well. It should be noted, as previously reported (Beushausen and Dales, 1985), that when oligodendrocytes are present at low density of 10^5 cells/well or less, JHMV replication is inefficient. This may explain the discrepancy in titers from astrocytes and oligodendrocytes.

b) fluid from cultures was sampled 24 hrs prior to and 24 hrs after treatment to determine released PFU.

c) note the large increase in titre during the 24 hr infection of astrocyte cultures.

various intervals for a period of 36 days. It is evident from Fig. 2.4 that in untreated control cells CNPase activity increased about threefold within 6 days and remained constant thereafter. By contrast, in the presence of the inducer dbcAMP, CNPase was at a much elevated value and continuously increased for over 30 days, up to the time sampling was terminated. Thus, in murine cultures containing oligodendrocytes dbcAMP enhanced many-fold CNPase activity, in the manner observed with rat material. With rat oligodendrocytes however the maximum induction occurs 15-21 days after treatment (Sprinkle et al., 1978; McMorris, 1983; Beushausen and Dales, 1985), whereas with murine cells the increase most probably continued beyond 30 days. Incidentally, murine cultures enriched for astrocytes, like their rat counterpart, are devoid of CNPase activity, so that the data from mixed cultures in Fig. 2.4 pertain only to oligodendrocytes.

The influence of cellular differentiation on replication of the two CV strains was tested by treating cultures enriched for astrocytes or oligodendrocytes with dbcAMP for various periods, followed by CV infection as indicated in Table 2.4. It is evident from the results summarized in this Table that exposure of astrocytes to dbcAMP for 9 or 14 days affected CV replication to a variable degree, reducing JHMV either not at all or to about 20% of the controls, while MHV_3

Figure 2.4. Time course for induction of CNPase activity in primary, dispersed glial cultures from DBA/2 mice. Treatment with dbcAMP and the initial sampling were undertaken at the time of explantation. Controls ●----●, dbcAMP treated o-----o. Two independent cultures were used to assay CNPase activity on the days indicated. However, since data were not obtained on the 35^{th} day in two other, comparable time-course experiments, the reliability of this value is uncertain and for this reason data for the 30^{th} and 35^{th} day are connected by a dashed line. Since the S.A. of CNPase was calculated on the basis of protein content in mixed cultures the actual S.A. in oligodendrocytes must have been greater.

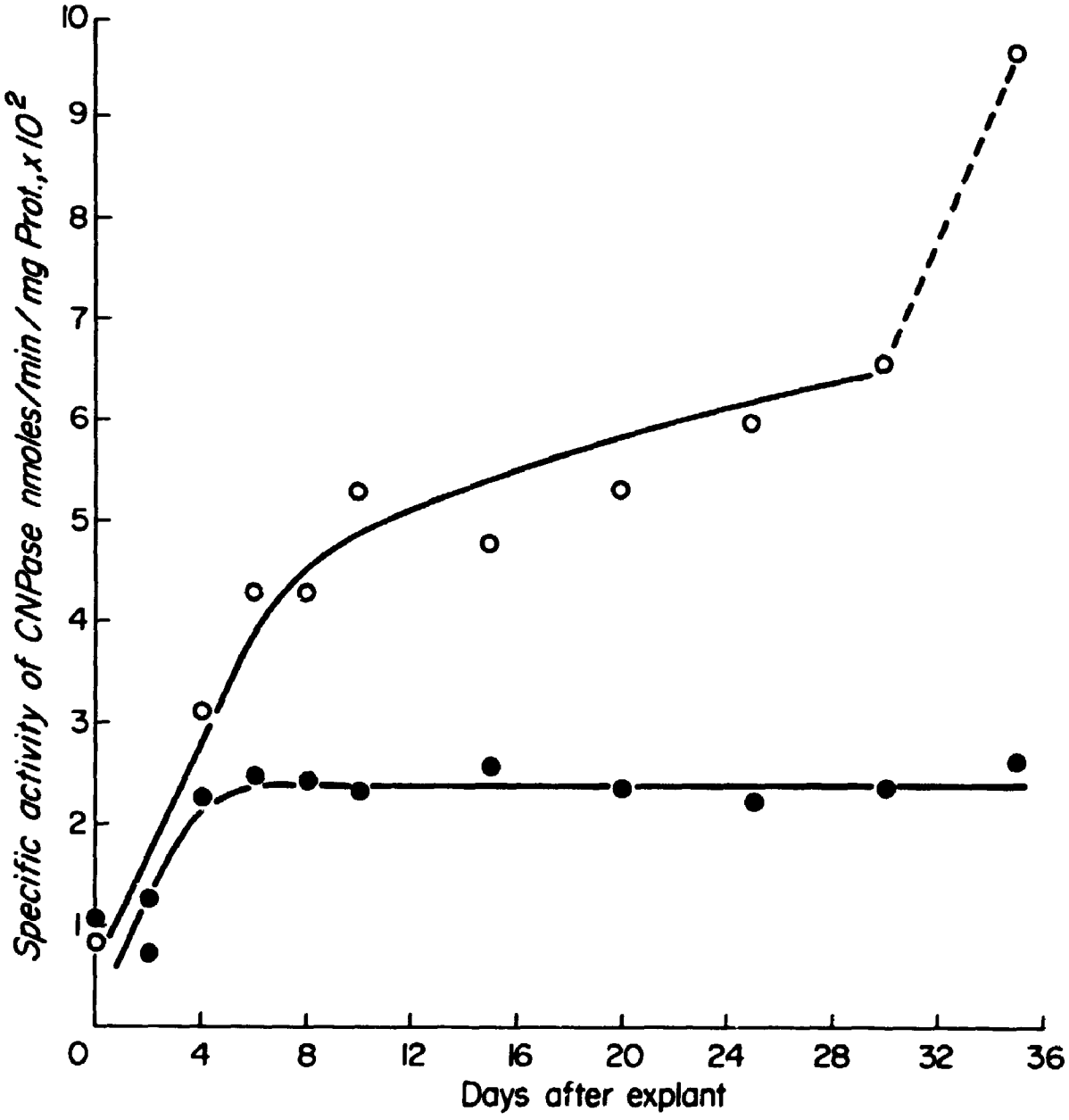

Specific activity of CNPase nmoles/min/mg Prot., x10²
10
9
8
7
6
5
4
3
2
1
0
4
8
12
16
20
24
28
32
36
Days after explant

Table 2.4

Effect of Treatment with dbcAMP on Viral Replication in CD.1 Mouse Astrocytes and Oligodendrocytes:

Days after treatment	JHMV x10^2 PFU/ml[a] control	JHMV x10^2 PFU/ml[a] 1 mM dbcAMP	MHV_3 x 10^2 PFU/ml control	MHV_3 x 10^2 PFU/ml 1 mM dbcAMP
		Astrocytes		
9	100	100	65	13
14	420	83	530	40
		Oligodendrocytes		
11	167	0.5	81	0.5
15	250	0	45	0
20	270	1	61	0.5
11 VSV*	300*	450*		

* oligodendrocyte culture infected with VSV

** the data shown in this table were derived from duplicate cultures and are representative of three reproducible experiments.

a) 24 hrs before the days indicated in the first column cultures were infected and then sampled 24 hrs later for PFU in the culture supernatant. The discrepancy in virus titers from oligodendrocyte culture shown in Tables 2.2-4 is most probably due to the much less efficient replication of virus in cultures of low density, containing 10^5 or fewer cells/well, as noted by Beushausen and Dales, (1985).

b) cells were allowed to attach for 24 hrs following isolation before commencing treatment with 1 mM dbcAMP. The dbcAMP containing or control medium was replaced every other day throughout the duration of the experiment. Cell density at the time of plating was approximately 1 x 10^5 cells/well.

yields were diminished to about 20 and 8%, respectively. By contrast either zero or almost no virus of either strain was generated by oligodendrocytes exposed to dbcAMP for 11, 15, or 20 days. Controls, using VSV showed that prolonged treatment of oligodendrocytes with dbcAMP had no effect on replication of this agent. Although the murine oligodendrocytes treated for 9 days or longer with dbcAMP were not checked for expression of CV antigens, surveys of rat oligodendrocytes revealed that treatment for 48 hr abolished production of JHMV antigens (Beushausen and Dales, 1985). Thus, in agreement with data from the rat model, coronavirus replication is blocked almost entirely in murine oligodendrocytes induced to differentiate in vitro, but is affected to a lesser degree in dbcAMP treated astrocytes.

2.4. DISCUSSION.

The present and other recent studies (Bologna-Sandru et al., 1981; Suzumura et al., 1984) show that it is possible to establish dispersed glial cultures containing oligodendrocytes from cerebral hemispheres of mice. As is the case with the rat (McCarthy and de Vellis, 1980; Pheiffer et al., 1981; Beushausen and Dales, 1985), the murine cells become organized into stratified layers consisting of tightly adherent astrocytes at the bottom and loosely attached oligodendrocytes on top. The differential adhesiveness enabled us to obtain cultures that are almost entirely astrocytes or highly enriched in oligodendrocytes. Challenge with the neurotropic CV-JHMV and viscerotropic MHV_3 showed that both strains can be replicated with equal facility in murine astrocytes and oligodendrocytes. This finding contrasts entirely with data from rat cells which show that tropism of JHMV is exclusive for oligodendrocytes and MHV_3 for astrocytes (Beushausen and Dales, 1985). Thus, species-related distinction between the in vitro infectability of specific cell types from the CNS has been demonstrated.

The species-related differences between the in vitro mouse and rat models can be correlated with the observed in vivo patterns of CNS neurological disease. These patterns are regulated by the age at the time of

challenge, route of inoculation, and genetic constitution of the rodent host and virus strain employed, as shown by previous work in our (Sorensen et al., 1980, 1982, 1984) and other laboratories (Nagashima et al., 1978a, 1978b, 1979; LePrevost et al., 1975; Stohlman and Weiner, 1981; Knobler et al., 1981b; Lavi et al., 1984; Weiner, 1973; Stohlman and Frelinger, 1978; Herndon et al., 1975; Levy-Leblond et al., 1979). Thus, in rats JHMV, but not MHV_3 can produce either an acute encephalomyelitis, if inoculated intracerebrally (ic) into very young animals, or a progressive paralytic disease with demyelinating lesions, if the agent is administered ic prior to weaning (Sorensen et al., 1982). In the mouse, however, both JHMV and MHV_3 induce neurological disease and, depending on the genetic endowment, this species may remain susceptible to CV even beyond 1 year of age (Lampert et al., 1973; Weiner, 1973; Herndon et al., 1975; Hirano et al., 1981; Stohlman and Weiner, 1981; Sorensen et al., 1982). Therefore, specificities of CV tropism manifested in tissue culture of glial cells appear to reflect accurately the events occurring within the CNS of the animal.

As previously demonstrated with material from the rat, addition of dbcAMP to murine oligodendrocytes induced in them an accelerated process of differentiation, manifested as elevation in CNPase activity (Sprinkle et al., 1978; McMorris, 1983; Beushausen and Dales, 1985), which is diagnostic of

myelin production. As found with rat cells, the process of murine oligodendrocyte differentiation is closely correlated with absence of virus production, in this case involving both JHMV and MHV_3. By contrast, dbcAMP treatment has much less influence on replication of these CV in mouse astrocytes, again consistent with the previous finding that this inducer has limited effect on production of MHV_3 in rat astrocytes (Beushausen and Dales, 1985). The observed reduction in virus yield from cultures of astrocytes treated with dbcAMP might be due to a slower effect on astrocyte differentiation thereby allowing production of CVs. Thus the current and previous observations indicate that infectability of the CNS of rodents is, at least in part, related to the maturation and differentiation of oligodendrocytes and, perhaps, astrocytes.

It should be mentioned that within 2 days after dbcAMP treatment JHMV production in rat oligodendrocytes ceases whereas at least 5 days of exposure to the inducer are required to inhibit MHV_3 or JHMV replication in mouse oligodendrocytes (data not shown). This finding might be related to differences in age between rats and mice when myelination is complete and also to the time interval when the animals remain susceptible to CNS disease caused by CVs. In mice the prolonged susceptibility to disease caused by infection is in line with an extended time period during which CNPase

induction and myelination occur (Kurihara et al., 1970; Morell et al., 1972; Barbarese et al., 1978) and is consistent with the long time required to obtain maximum CNPase induction in vitro as shown in Fig. 2.4. By contrast, the unusually sensitive WF rats remain susceptible for only about 15-21 days, to disease caused by JHMV (Sorensen et al, 1980), which is coincident with the completion of myelination and CNPase induction in vivo (Sprinkle et al., 1978) and in vitro (Abney et al., 1981; Barbarese and Pfeiffer, 1981; McMorris, 1983). These findings focus further on the possible relevance of age-related oligodendrocyte maturation in the CNS and susceptibility to disease within the CNS caused by CVs.

It should be kept in mind that the infectability of oligodendrocytes by these agents cannot account entirely for the CNS diseases observed, since the ability of CVs to replicate in neurons and other cell types has been abundantly documented (Nagashima et al., 1978; Knobler et al., 1981a; 1981b; Dubois-Dalc et al., 1982; Collins et al., 1983; Sorensen et al., 1984; Beushausen and Dales, 1985). Recent investigations using cDNA probes have, in fact, shown by in-situ hybridization that latent JHMV can be maintained for prolonged periods in the cytoplasm of neurons found within specific areas of the rat brain, particularly in the hippocampus (Sorensen and Dales, 1985). This suggests that neurons may function as repositories of latent and persistent CV

infections. The relative importance of oligodendrocytes versus the other neural cell types in the disease process must await future elucidation.

In conclusion, CV challenge of murine glial cells in vitro appears to accurately reflect the type of infections observed in vivo. In addition, differentiation of mouse glial cells appears to restrict CV replication which is consistent with previous observations in rat oligodendrocytes (Beushausen and Dales, 1985).

We will examine the influence of glial cell differentiation on CV replication more closely in the next chapter where evidence is presented outlining one possible mechanism accounting for these observations.

CHAPTER 3

RELATIONSHIP BETWEEN SUPPRESSION OF CORONAVIRUS JHMV REPLICATION IN DIFFERENTIATED PRIMARY GLIAL CELLS AND INHIBITION OF NUCLEOCAPSID PROTEIN DEPHOSPHORYLATION

3.1. INTRODUCTION.

Development of neurological diseases in rodents following intracerebral (IC) challenge with coronavirus (CV) JHMV is an age-related process with resistance manifesting itself in Wistar Furth (WF) rats at about the time of weaning 3 weeks post-birth (Sorensen et al., 1980). Differentiation of primary rodent explant cultures of oligodendrocytes in vitro, using the metabolite dibutyryl cAMP (dbcAMP) which raises intracellular concentrations of cAMP, allowed us to more selectively demonstrate induction of resistance to JHMV with maturation of this cell type (Beushausen and Dales, 1985; Wilson et al., 1986).

Treatment of host-cells with metabolites which increase the intracellular concentration of cAMP, affects not only CV replication but also that of other viruses in either a suppressive (measles) or enhancing (rubella and polyoma) manner (Miller and Carrigan, 1982; Robbins and Rapp, 1980; Van Alstyne and Paty, 1983; Baur and Manor, 1988).

The regulatory effects of increasing intracellular

concentrations of cAMP are thought to be mediated through induction of the cAMP-dependent protein kinases types 1 (PK_1) and 2 (PK_2) (Flockhart and Corbin, 1982; Nimmo and Cohen, 1977; Rubin and Rosen, 1975). Analysis of WF primary oligodendrocytes induced to differentiate with dbcAMP failed to show an induction of PK_1 or PK_2 activity, but did show an induction of the regulatory subunit (R_1) from PK_1 (Beushausen et al., 1987). While the only known function of R_1 at this time is to complex with the catalytic subunit thereby controlling its phosphorylating activity, previous reports employing R_2 from PK_2 indicate a possible second function involving inhibition of phosphoprotein phosphatases (PPPase) (Jurgensen et al., 1985; Khatra et al., 1985). Previous evidence from this laboratory indicates the involvement of a PPPase during the early stages of CV infection (Beushausen et al., 1987) possibly being responsible for nucleocapsid protein (NC) dephosphorylation and/or processing of this protein to lower molecular weight (MW) forms. These events are thought to occur during the uncoating stage of virus infection after uptake from the cell surface but before release of the viral genome into cytosol.

These early stages of CV infection in differentiated glial cells are of particular interest since previous work has demonstrated a block in the CV replication cycle occurring after virus uptake from cell

surface, via receptor-mediated endocytosis, but before synthesis of viral RNA (Bueshausen et al., 1987). The sequence of events after adsorption and before release of the genome into the cytosol, possibly occurring within host-cell endosomes, are thought to be essential for initiation of CV infection.

This study was undertaken to more precisely define the possible role, if any, that R_1 might have in restricting CV-JHMV replication within host cells involving the specific inhibition of a phosphoserine PPPase found within the endosome fraction of cells.

3.2. MATERIALS AND METHODS.

3.2.1. Continuous Line of Cells.

L-2 mouse fibroblasts (Rothfels et al., 1959) were cultured as previously described in Chapter 2 in Eagle's minimal essential medium (Flow Laboratories, Inc.) containing 8% Nu-serum (Collaborative Research, Inc.) plus 2% heat-inactivated fetal bovine serum (Bocknek Laboratories) termed complete medium (CM).

3.2.2. Primary Rat Glial Cultures.

Cultures of primary WF oligodendrocytes were isolated essentially as previously described in Chapter 2 from mixed cultures of glial cells obtained by explanting the cerebral cortices of neonatal rats. Briefly, the cerebral hemispheres from 6 neonatal WF rat pups were excised, the meninges removed, and placed in ice-cold BME_{10} medium. Hemispheres were washed three times with 10 mls BME_{10} to remove any remaining meninges and then titurated through 10 ml pipette to produce a homogeneous suspension. Cells pelleted by centrifugation at 1000 x g for 5 min were resuspended in 9 mls of BME_{10} and plated into 175 cm^2 tissue culture flasks (Nunc), in total volume of 30 mls BME_{10}. The flasks had been treated for 1 hour (hr) with 50 ug/ml poly-L-lysine (Gibco), > 200,000 mw) at 22 C prior to use. Cultures were maintained for 5 days at 37 C in humidified atmosphere before changing medium. On day 7

post-explantation cultures were used for oligodendrocyte isolation as previously outlined in Chapter 2.

3.2.3. Treatment of Cells with Protein Kinase Inhibitors.

Cultures of oligodendrocytes at 5 X 10^5 cells per well in 24 well plates were pre-treated for 3 days at 37 C with BME_{10} medium either alone or in conjunction with dibutyryl cAMP (dbcAMP) (Sigma) and/or protein kinase inhibitors H8 and HA1004 (Seikagaku Kogyo Co.). Cultures were then infected with JHMV at an MOI of 1 PFU per cell for 1 hr at 37 C. Cells were washed three times with 1 ml PBS (pH 7.2) before incubating cultures with appropriate medium at 37 C for various intervals. Medium was changed daily following plaque assay for virus production as previously described (Chapter 2).

3.2.4. Photoaffinity Labeling of R_1 Protein From Glial Cells.

The presence of R_1 protein in mixed WF glial cultures was determined using the photosensitive cAMP analogue 8-azido-[^{32}P]cAMP (67.6 Ci/mmole, 14 nM/ml, ICN) according to Walter et al., (1979) with minor alterations.

Cultures of mixed WF glial cells, grown in 25 cm^2 tissue culture flasks (Nunc), were harvested by trypsinizing monolayers with 0.1% trypsin (Gibco) in PBS solution. Cells were pelleted by centrifugation at 150

x g for 5 min and resuspended in 600 ul of cold extract buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 5 ug/ml PMSF. Cells were then incubated for 10 min on ice and extracts prepared by passaging the suspension ten times through 30 gauge needle on 1 ml syringe on ice. Cellular debris was pelleted by centrifugation at 15,000 x g 15 min at 4 C and cytosol fraction removed and stored -20 C until use. Protein determinations were carried out using a Bio-Rad protein determination kit with BSA as standard.

Covalent coupling of [^{32}P]-labeled cAMP analogue to R_1 protein was carried out as follows. Reactions were initiated by incubating 6 ug of glial cell extract (60 ul volume) with 60 ul hybridization buffer (HB), containing 50 mM MES (pH 6.2), 10 mM $MgCl_2$, 1 mM EGTA, 50 ug/ml PMSF and 6 pM of 8-azido cAMP (4 ul of 8-azido stock diluted in 560 ul HB) for 1 hr 22 C in the dark. Reaction mixtures were then irradiated on ice 10 min with UVS-11 hand lamp at a distance of 10 cm from eppendorf tubes. UV light covalently couples the 8-azido cAMP analogue to binding sites on R_1/R_2 proteins. Following irradiation, 30 ul of 3 X dissociation buffer (Laemmli, 1970) was added and tubes boiled 3 min followed by quenching on ice. Proteins were then electrophoretically separated on 10.5% SDS-PAGE. Gels dried under vacuum and R_1/R_2 visualized by autoradiography using Kodak X-OMAT X-ray film (Dupont)

at -70 C.

3.2.5. Isolation of NC Protein from Infected Cells.

The isolation of NC protein from infected L-2 mouse fibroblasts was performed essentially as previously described (Mohandas and Dales, 1989). Briefly, approximately 4-5 X 10^7 L-2 cells in large tray (Nunc) were adsorbed with JHMV at an MOI of 1 for 1 hr at 22 C with rocking. Inoculum was removed and cultures incubated at 37 C with CM until 90-100% syncytia formation. Supernatant was then removed and cells washed twice with 50 mls cold PBS. To the monolayer was then added 3 mls of cold distilled water, monolayer scraped off and resuspended by passaging five times through 18 gauge needle on 5 ml syringe on ice. The resulting suspension was passaged ten times through 30 gauge needle on 5 ml syringe to disrupt cells.

The homogenate was mixed with volume of 10 X TMEN-6 buffer containing 0.5 M Tris-maleate (pH 6.0), 0.01M EDTA and 1.0 M NaCl, to give 1 X TMEN-6 concentration, usually about 0.7-0.8 mls. The suspension was vortexed, centrifuged at 6000 x g for 15 min 4 C and supernatant removed. NP-40 was then added to a final concentration of 0.25% and suspension manually mixed twenty-five times before passing mixture through 26 gauge needle 10-15 times. The mixture was incubated on ice for 30 min before proceeding to sucrose gradients. Following incubation, 2 mls of NP-40 supernatant was loaded onto a 15 to 50%

w/v sucrose gradient consisting of 1 ml 65% sucrose in TMEN-6 plus 4.5 mls each of 15 and 50% sucrose in TMEN-6 containing 0.1% NP-40, and centrifuged at 130,000 x g for 18 hr 4 C in SW-41 rotor. Fractions (1 ml) were collected from bottom of tubes following centrifugation and 25 ul samples analysed by 10% SDS-PAGE. Peak fractions containing NC p56 protein (usually fractions 1-4) were then pooled and run on 10% preparative PAGE gel. Bands were visualized with 4M sodium acetate, excised, and protein extracted by homogenizing gel in ammonium bicarbonate buffer containing 5 mM ammonium bicarbonate, 20% SDS in H_2O. The resulting suspension was mixed on a rotary shaker for 18 hr at 22 C before harvesting supernatant by pelleting out remaining gel pieces at 6000 x g for 15 min 4 C. The supernatant was then dialyzed against dH_2O approximately 18 hrs with 2 changes of distilled water. The NC solution was then aliquoted and frozen -20 C.

3.2.6. Isolation of Endosomes.

Suspension culture of L cells at approximately 1-2 x 10^6 cells/ml, total of 1-2 x 10^8 cells, was concentrated by centrifugation at 800 x g for 10 min. The cell pellet was washed three times with homogenization buffer (HB) containing 0.25 M sucrose, 1 mM EDTA (pH 6.8), by resuspending and pelleting cells at 800 x g for 10 min. After final wash the pellet was resuspended in HB and homogenized in dounce homogeniser until

approximately 90% cell breakage as monitored under phase contrast optics for intact cells. The resulting suspension was centrifuged at 800 x g for 10 min to clarify supernatant and then loaded onto a percol gradient containing 3 mls 60% sucrose, 25 mls percol, 3 mls HB and 2 mls sample (density approximately 1.070 gms/ml) in SW28 tubes. The gradients spun at 20,000 x g for 2 hrs 4 C before collecting 1 ml fractions from bottom of tubes. Fractions were assayed for acid phosphatase activity and peak fractions containing endosomes with some remaining plasma membrane run through a second percol gradient consisting of 1 ml 60% sucrose, 9 mls percol, 2 mls sample (density approximately 1.045 gms/ml) in SW-41 tubes and centrifuged at 20,000 x g for 50 min 4 C. Fractions were again collected from bottom of tube and assayed for acid phosphatase activity. Peak acid phosphatase fractions were collected, pooled, and used as endosome enriched preparations.

3.2.7. Acid Phosphatase Enzyme Assay.

To identify those fractions from cellular homogenates containing endosomes, acid phosphatase assays were carried out essentially as described by Pallen and Wong (1983) and outlined below. Samples from the percol fractions containing 50 ug of protein were mixed with Acid Phosphatase buffer containing 0.25 mM Tris-MES-Acetate (pH 5.5), 2.5 mM $MnCl_2$, 0.1% Triton,

2mM para-Nitrophenyl phosphate (pNPP) substrate and water to 1 ml volume and incubated at 30 C for 60 min. The reactions were terminated by addition of 50 ul of 13% K_2HPO_4, and incubated a further 15 min 22 C to allow colour development. Reactions were then centrifuged at 1000 x g for 10 min and adsorbance measured at 410 nm wavelength on a spectrophotometer.

3.2.8. Isolation of Catalytic and Regulatory Subunit From PK Holoenzyme.

The methodology was essentially as outlined in Beavo et al. (1974), using R_1 affinity columns to separate the holoenzyme components. Briefly, PK_1 holoenzyme preparations from rabbit skeletal muscle (Sigma) were reconstituted in dH_20 at a concentration of 5 mgs/ml and loaded onto a N6-2C-cAMP Sepharose column (5 ml packed volume) which had been pre-equilibrated with MES buffer (pH 6.5) containing 5 mM MES, 9 mM NaCL, 15 mM 2-mercaptoethanol. The column was washed with a large volume of 2 M NaCl in MES buffer followed by MES buffer at 22 C. The R_1 was then eluted by incubating the gel at 30 C in 3 volumes of 30 mM 3':5'cAMP (Sigma) in MES buffer for 1 hr. The supernatant containing R_1 was concentrated using Amicon YM-10 membrane and dialysed for 2 days with 4 X 1L changes of MES buffer at 4 C. Protein determinations were performed using Bio-Rad protein determination Kit with BSA as standard.

A second isolate of R_1 from WF rat skeletal muscle was a gift from Dr. B.D. Sanwal's laboratory, Dept. of Biochemistry, University of Western Ontario. Catalytic subunits collected in the void volume following passage through R_1 affinity column were also concentrated on Amicon YM-10 membranes and dialysed as outlined above.

3.2.9. Protein Kinase Assay.

Following isolation of regulatory and catalytic subunits from PK_1 holoenzyme, samples of each were assayed for residual PK activity using histone IIA (Sigma) as substrate. The procedure was essentially as described by Corbin et. al., (1974) and outlined below. Equal volumes of 84 mM KH_2PO_4 buffer (pH 6.8), 28 mM Mg acetate, 1.75 mM ATP, histone IIA at 50 mg/ml, 10 uM 3':5'cAMP and dH2O were mixed, termed solution A, and to this mixture was added 50 uCi [^{32}P]-ATP (Amersham sp. act. > 75,000 Ci/mmol). To test for PK activity 50 ul aliquots of solution A were mixed with approximately 3 ug of protein of each sample in glass tubes. The reactions were incubated for 15 min at 30 C before being spotted onto GF/C glass fibre filters (Millipore) and the reaction terminated by placing filters in 10% ice cold Trichloroacetic acid (TCA) for 1 hr with shaking. The TCA solution was changed three times with 25 mls in a glass beaker before washing filters twice with 100% ethanol and drying at room temperature. Filters were then counted

with 3 mls omnifluor in a scintillation counter.

3.2.10. Phosphoprotein Phosphatase Assay.

Phosphoserine phosphatase activity against JHMV NC protein was assayed essentially as previously described (Mohandas and Dales, 1989) with minor changes. Reactions were initiated containing 25 mM Tris-maleate (pH 7.0), 25 mM $MgCl_2$, 1% Triton X-100, 25 ul endosome or homogenate extract (approximately 25 ug protein) and 40 ul NC substrate (^{32}P-labeled or native), for 90 min at 30 C. Reactions were terminated by addition of 50 ul of 1 % BSA and 400 ul of 25% TCA for determination of solubilized counts or 50 ul of 3 X Laemmli Disscciation buffer (DB) (Laemmli, 1970) if samples were used for SDS-PAGE analysis.

The amount of specific NC dephosphorylation can be calculated by determining the percentage of [^{32}P]-released into the supernatant following PPPase assay essentially as outlined by Maeno and Greengard (1972). Following addition of 1% BSA and 25% TCA as outlined above, reactions were incubated for 1 hr on ice and then centrifuged at 15,000 x g for 10 min 4 C. Supernatant aliquots, 500 ul, were then mixed with 50 ul of 0.01 M KH_2PO_4 and 150 ul of 5% ammonium molybdate. The phosphomolybdate complex was then extracted by addition of 1 ml isobutyl alcohol. Aliquots of this mixture were counted with 3 mls Aquamix (ICN) in scintillation counter

using identical quantities of undephosphorylated NC protein as standard for total amount of [^{32}P]-label.

Additional assays using PK holoenzymes or their consitituent subunits were set up essentially as described with the addition of 1 mM 3':5' cAMP and 3 ug of either PK holoenzyme or R_1/C subunits. The methodology of these assays was also slightly modified with a 30 min pre-incubation of all components at 30 C minus the [^{32}P]-NC substrate to allow interaction of endosomes and PK components. Following pre-incubation [^{32}P]-NC was added and reaction continued at 30 C for further 90 min before processing as previously outlined above.

3.2.11. Western Immunoblotting.

Specific staining of JHMV NC protein by Western immunoblotting which has been previously described (Coulter-Mackie et al., 1985) was carried out following separation of proteins on 10.5% SDS-PAGE. Following separation proteins were electrophoretically transferred onto 0.22 um nitrocellulose sheet (Schleichter and Schuell) for 18 hr at 4 C (6 v/cm) using Bio-rad transfer apparatus. Nitrocellulose was then removed, stained 10 min with 0.1% amido black in 50% methanol, 10% acetic acid and destained for 20 min with 3 changes of 50% methanol, 10% acetic acid. The blot was then incubated for 1 hr in PBS plus 0.05% Tween-20 at 22 C with at least 6 changes of solution. Labeling of

proteins with mouse Mab against JHMV NC protein (Dr. M. J. Buchmeier, Scripps Clinic and Research Foundation, La Jolla, California) was then initiated for 1 hr 22 C using 1 ug of Mab (1 ul of Mab stock) per lane in PBS + 0.05% Tween-20. The blots were washed six times with PBS + 0.05% Tween-20 for 1 hr total before incubating 1.5 hrs 22 C with 1 ug/lane of rabbit anti-mouse kappa chains (Miles Laboratories). The blots were again washed six times for 1 hr total with PBS-Tween-20 and subsequently labeled 1 hr 22 C with [^{125}I]-Protein A (5 uCi/ug, NEN-Dupont) at approximately 1 x 10^5 cpm per lane in PBS-Tween-20. Blots were then washed, dried on filter paper, and exposed for autoradiography using Kodak X-Omat X-ray film (Dupont) at -70 C.

3.2.12. Nucleic Acid Binding Assay.

To determine if dephosphorylation of NC protein had any affect on its ability to bind nucleic acids an in vitro nucleic acid binding assay was employed, essentially as described by Robbins et al., (1986). PPPase assays were terminated by addition of 3X DB and proteins separated on 10.5% SDS-PAGE. Gel contents were then transferred onto 0.22 um nitrocellulose (Schleicher and Schuell) blots for 18 hrs at 4 C (40 volts) using transfer apparatus (Bio-Rad). Proteins were visualized by amidoblack staining and the blot was equilibrated for 1.5 hrs 22 C with standard binding buffer (SBB) containing 10

mM Tris-HCL (pH 7.0), 0.05 M NaCL, 1 mM diNa-EDTA, 0.02% bovine serum albumin (BSA) and 0.02% Ficoll paque. Blot then probed for 1.5 hrs at 22 C with [^{32}P]-labeled CV specific plasmid g344 (Budzilowicz et al., 1985) DNA, at approximately 5 x 10^5 cpm/lane in SBB buffer. Following labeling blots were washed six times with SBB for 1 hr total 22 C, dried on filter paper and exposed to X-Omat X-ray film (Dupont) at - 70 C.

3.2.13. Isolation and Labeling of Plasmid DNA.

Plasmid was labeled by nick translation essentially as described by Rigby et al., (1977) and purified using a Sephadex spin column (Maniatis et al., 1982). Reactions were prepared by mixing 1 ug of plasmid DNA, 1 ug of 10 ug/ml DNAase 1 and 25 units of DNA polymerase 1 (Klenow fragment) with reaction buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 50 ug/ml BSA, 3 ug/ml dTTP, 3 ug/ml dGTP, 13 mM 2-mercaptoethanol, 250 uCi [^{32}P]-dCTP and 250 uCi [^{32}P]-dATP (600 Ci/mM, ICN). Mixture was first incubated at 15 C for 90 min before reaction stopped by addition of 15 ul of 0.5 M EDTA (pH 7.4) and incubation continued a further 10 min at 68 C. Labeled plasmid DNA was separated from free nucleotides as described by Maniatis et al. (1982) using a Sephadex G-50 column. Briefly, labeled DNA in 100 ul volume (volume made up with TE buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) was

added to a 0.9 ml packed volume Sephadex G-50 column in 1 ml syringe which had been pre-equilibrated with STE buffer containing 0.02 M Tris-HCl (pH 7.4), 10 mM EDTA and 0.1 M NaCl. The column was then centrifuged at 1500 x g for 4 min 22 C with the effluent collected into an eppendorf tube on the end of syringe. DNA was precipitated for 18 hr at -20 C with 50 ug yeast tRNA, 12 uls of 3 M NaCl and 300 uls of 95% ethanol, followed by centrifugation at 15,000 x g for 15 min. The pellet was resuspended in 300 ul TE buffer and an aliquot of the DNA solution (1 ul) was counted by spotting onto a glass fibre disc with radioactivity determined using a scintillation counter. The remainder of plasmid was frozen -20 C until use. Prior to hybridization reaction the probe was denatured by boiling for 5 min followed by quenching on ice.

3.3. RESULTS.

3.3.1. Replication of JHMV in WF Oligodendrocytes Pretreated With dbcAMP and PK Inhibitors.

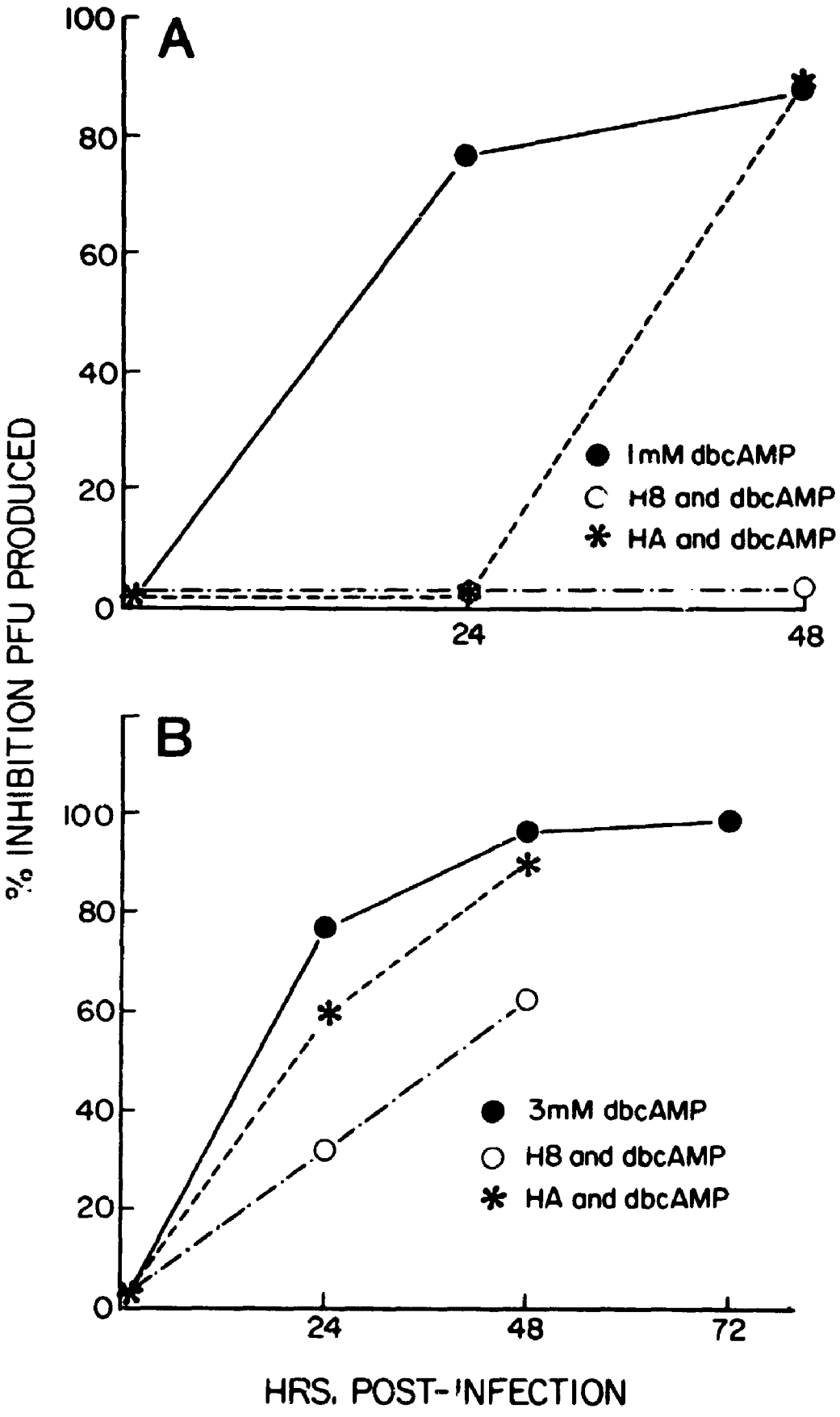
If, as indicated by previous data (Beushausen and Dales, 1985; Wilson et al., 1986), the modulation of cAMP dependent-PK(s) influences oligodendrocyte differentiation and coincidentally inhibits coronavirus expression, one might suppose that inhibitors of PK activity should counteract this effect. This notion was tested by pre-treating WF oligodendrocyte cultures with isoquinoline sulfonimide derivatives H8 and HA1004, compounds known from previous studies to inactivate cAMP-dependent PKs by binding to cataytic subunits (Hidaka et al., 1984).

In confirmation of our previous findings treatment of primary oligodendrocytes with 1 mM dbcAMP suppressed JHMV replication by 80% within 24 hr and over 90% in 48 hr (Figure 3.1 A). At 3 mM dbcAMP virus suppression was almost complete in 48 hr (Figure 3.1 B). Simultaneous exposure of oligodendrocytes to 1 mM dbcAMP and 25 uM H8 facilitated the same rate of virus production for 48 hr as found in untreated controls (Figure 3.1A). If dbcAMP was increased to 3 mM, addition of 25 uM H8 alleviated the inhibition only partially, as shown in Figure 3.1B. The other inhibitior of PK, HA1004, when added at 50 uM with 1 mM dbcAMP was effective during the initial 24 hr

Figure 3.1. Effect of protein kinase inhibitors on JHMV replication in cultures of glial cells induced to differentiate with dbcAMP.

A) cultures of glial cells at 2.5 x 10^5 cells per cm^2 were pre-treated for 3 days with 1 mM dbcAMP ---- ● ----, 1 mM dbcAMP + 25 uM H8 ---- o ----, or 1 mM dbcAMP + 50 uM HA1004 -- - * - --, prior to inoculation with JHMV MOI 1. The culture fluid was assayed for PFU at times indicated. Data are expressed as the % of virus produced compared to that by controls in BME_{10} only.

B) experimental conditions as in A) except that dbcAMP was added at 3 mM.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0
24
48
72
3mM dbcAMP
H8 and dbcAMP
HA and dbcAMP
% INHIBITION PFU PRODUCED
HRS. POST-INFECTION

but not later (Figure 3.1A). Cotreatment of cells with 3 mM dbcAMP and 50 uM HA1004 was virtually ineffective at reversing CV suppression (Figure 3.1B).

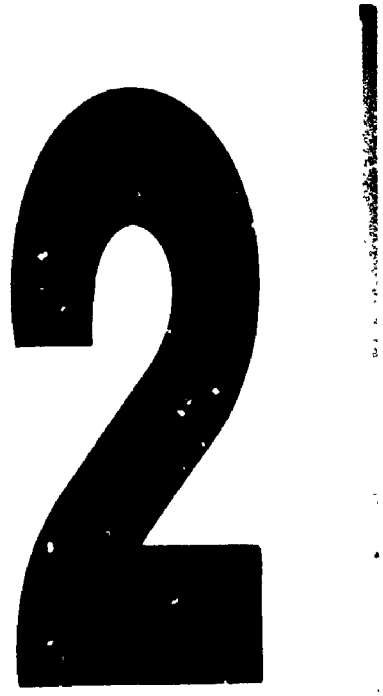
These findings offer suggestive evidence supporting the notion that PK(s) influenced by elevated cAMP levels in differentiating oligodendrocytes may somehow be connected with inhibition of JHMV expression.

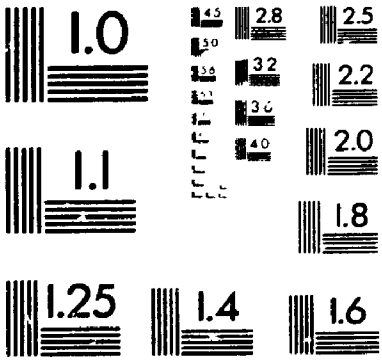
3.3.2. Effects of PK Inhibitors on R_1 Induction in Glial Cells Simultaneously Treated with dbcAMP.

The previous section demonstrated the ability of PK inhibitors to reverse the inhibition of JHMV replication in cultures of oligodendrocytes induced to differentiate with dbcAMP. We therefore wanted to determine if PK inhibitors could directly interrupt the induction of R_1 protein previously observed in differentiating glial cells (Beushausen et al., 1987). For these experiments, cultures of mixed WF glial cells containing oligodendrocytes and astrocytes were pre-treated with dbcAMP and PK inhibitor H8 for 3 days and then assayed for quantities of R_1 protein present by photoaffinity labeling cell extracts with 8-azido [^{32}P]-cAMP.

The induction of R_1 protein in dbcAMP treated cultures as compared to BME_{10} treated controls (Figure 3.2, lanes 4 and 3) corroborates previous data on R_1 induction with glial cell maturation (Beushausen et al., 1987). The increase corresponds to approximately a 40

Figure 3.2. Effects of PK inhibitors on R_1 induction in glial cells simultaneously treated with dbcAMP. Mixed cultures of WF glial cells, treated with dbcAMP and protein kinase inhibitor H8, were monitored for R_1 induction 3 days post-treatment by photoaffinity labeling cell extracts with 8-azido [^{32}P]-cAMP. Autoradiogram showing in lanes 1 and 2, PK_1 and PK_2 respectively; lane 3, untreated controls; lane 4, cells treated with 1 mM dbcAMP; lane 5, cells treated with 25 uM H8; lane 6, cells treated with 25 uM H8 plus 1 mM dbcAMP; lane 7, cells treated with 13 uM H8 plus 1 mM dbcAMP.

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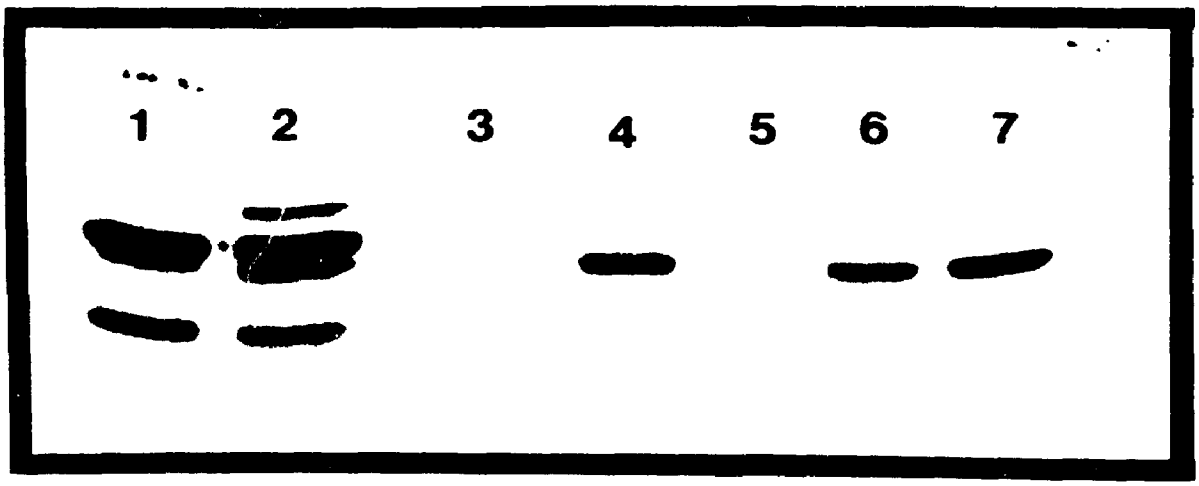
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fold induction of R_1 protein as determined by densitometric scanning of autoradiogram. Cultures treated with 25 uM H8 alone (lane 5) show no induction in R_1 levels being comparable to BME_{10} controls. Glial cells simultaneously treated with 25 uM H8 and 1 mM dbcAMP however, do show an induction in R_1 levels with protein levels about 60% that of cultures treated with dbcAMP alone (lane 4). 25 uM H8 was therefore not completely effective in inhibiting R_1 induction in mixed glial cells treated with dbcAMP. Glial cultures concomitantly treated with 13 uM H8 and 1 mM dbcAMP (lane 7) again show induction of R_1 with levels being comparable to cultures treated with dbcAMP alone.

3.3.3. Effects of cAMP Dependent Protein Kinases and Their Subunits on Endosomal Phosphoprotein Phosphatase Activity With JHMV Nucleocapsid Protein.

Presence of a phosphoserine phosphoprotein phosphatase in endosomal fractions, active against the phosphorylated nucleocapsid of JHMV, which has been previously documented (Mohandas and Dales, 1989), drew our attention to the possible role of this enzyme in the uncoating process of coronaviruses. Since differentiation inducers such as cAMP cause modulation in PK expression, including induction of R_1 protein from type 1 cAMP dependent PK, and affect JHMV replication within oligodendrocytes at an early stage subsequent to

uptake (Beushausen and Dales, 1985; Beushausen et al., 1987), it was reasoned that PK or subunits thereof might affect the PPPase directly. In this regard it should be remembered that there is an intimate interplay between PKs and PPPases in the metabolic pathways in which these enzymes function (Ingebritsen and Cohen, 1983).

As explained above, the present study is based on the use of endosomes from L cells because this cell fraction could be isolated reproducibly and routinely as a defined fraction, providing an adequate source of PPPase with high specific activity.

The results (Table 3.1) illustrate the ability of PK_1 and PK_2 holoenzymes to inhibit JHMV nucleocapsid dephosphorylation only in the presence of exogenous cAMP. The presence of cAMP in the reaction mixture promotes dissociation of PK holoenzymes into their constituent components resulting in free R and C subunits. When enriched fractions of R_1 and C subunits were subsequently tested, inhibition of NC dephosphorylation was confined to the R_1 subunit (Table 3.1). Thus, inhibition of PPPase activity is specific for the regulatory subunits of both PK_1 and PK_2. The ability of R_1 to inhibit PPPase activity in the absence of cAMP is only marginally affected (Table 3.1) probably due to R_1 being previously complexed with cAMP during isolation. Confirmation of R_1 inhibition of NC dephosphorlyation was obtained by separating reaction

Table 3.1

Effects of Addition of PK and Subcomponents on Rate of NC Dephosphorylation by Endosomal PPPase:

Addition	SA dpm [^{32}P] released per mg Protein[a]	%Inhibition ± S.E.[b]
none	1,440 (9)	
PK_1 Holoenzyme (+) cAMP	1,240 (5)	14 ± 3 ?
PK_1 Holoenzyme	1,480 (4)	0
PK_2 Holoenzyme (+) cAMP	1,120 (4)	22 ± 2.6
PK_2 Holoenzyme	1,480 (2)	0
R_1 3 ug (+) cAMP	760 (9)	47 ± 7
R_1 3 ug (-) cAMP	960 (3)	33 ± 2.7
C_1 5 ug (+) cAMP	1,680 (4)	0

a) Representative experiment with number of repetitions in paranthesis. Each experiment was done in triplicate. One unit of specific activity (SA) is defined as 1% of [^{32}P] released in 90 min at 30 C.

b) % inhibition ± standard error for number of experiments in parenthesis.

components on 10.5% SDS-PAGE followed by autoradiography (Figure 3.3).

In addition, the effects of increasing R_1 concentrations on PPPase activity was examined (Figure 3.4). Increasing R_1 concentrations beyond 3 ug had only a marginal effect on PPPase activity (Figure 3.4) and thus the remainder of these studies will use R_1 at 3 ug concentration.

It should be noted that R_1 protein from both rat and rabbit skeletal muscle were tested and found to be equally effective in inhibiting the endosome fraction PPPase (data not shown).

3.3.4. Proteolytic Cleavage of NC during PPPase Assay.

Infection of host cells with CV often results in several lower molecular weight proteins which are related to P56 as demonstrated by monoclonal antibodies and 2-D trypic peptide digests (Cheley and Anderson, 1981; Coulter-Mackie et al., 1985). These lower molecular weight proteins are thought to be NC breakdown products resulting from proteolytic digestion of P56 molecules not complexed with viral genomes. To examine whether dephosphorylation within the endosome environment was sufficient to allow breakdown of P56, PPPase assays using L cell endosomes were undertaken and reaction products analysed by autoradiography following separation of components on 10% SDS-PAGE and transfer to

Figure 3.3. Autoradiographic demonstration of the inhibitory effect of R_1 subunit on the endosomal PPPase. The bands mark [^{32}P]-associated with NC protein of JHMV. Lane 1, NC in the absence of endosome PPPase; lane 2, NC following reaction with PPPase for 90 min. at 30 C; lane 3 as in lane 2 but in the presence of 3 ug R_1.

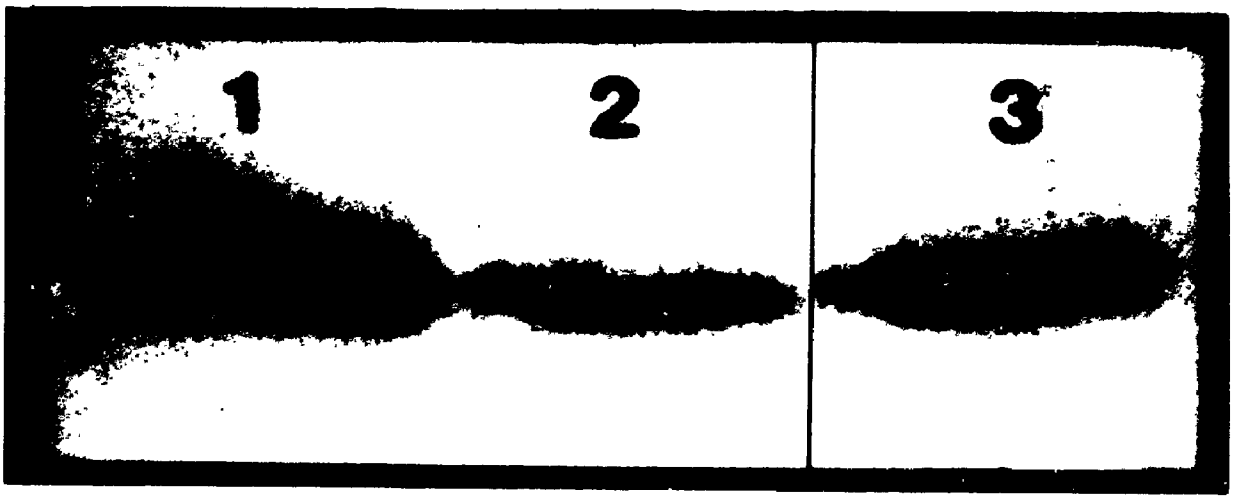
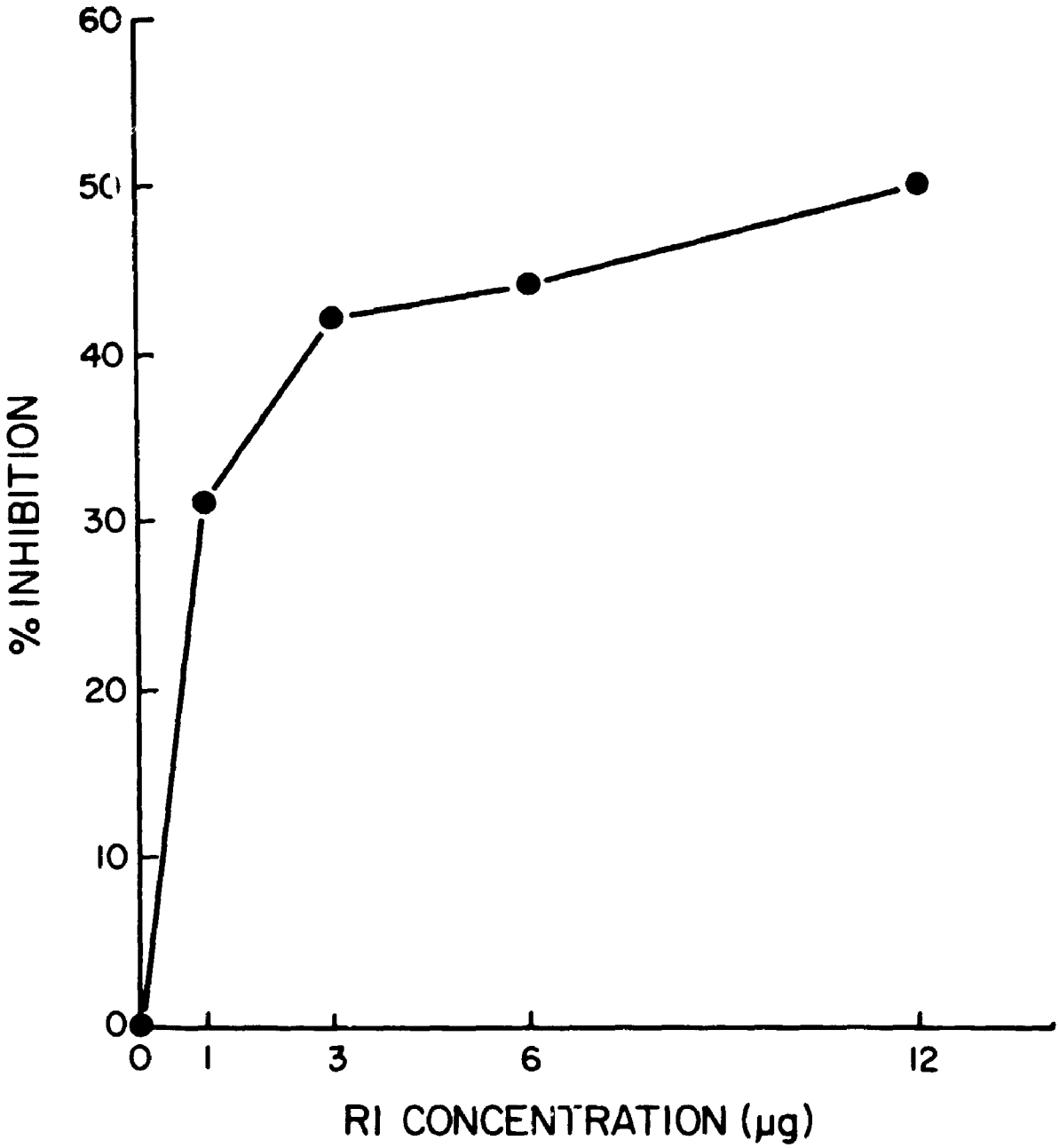

1
2
3

Figure 3.4. Inhibition of NC protein dephosphorylation by endosomal PPPase as a function of increasing R_1 concentration.


60
50
40
30
20
10
0
% INHIBITION
0
1
3
6
12
RI CONCENTRATION (µg)

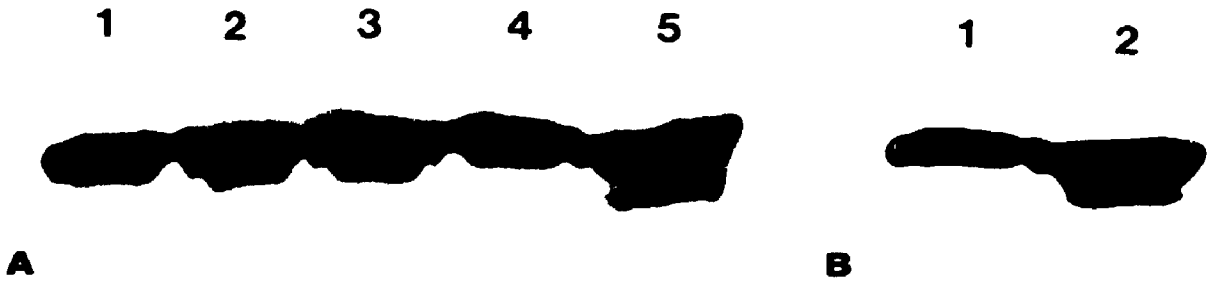
nitrocellulose for Western blotting. The results of Western blotting show no significant change in MW of P56 or any evidence of breakdown products following dephosphorylation by endosome PPPase (Fig. 3.5A lanes 1-3).

When similar experiments were done however, using whole cell extracts from either L cells (lane 5) or WF brains (Fig. 3.5B, lane 2) we were able to demonstrate, in addition to dephosphorylation, the conversion of P56 to lower MW P50. Thus, dephosphorylation within endosomes is not sufficient for breakdown of P56. The conversion of P56 to lower MW species probably requires a protease present within the cytoplasmic environment of host cells. Whether dephosphorylation of native P56 is required before processing can occur within the cytoplasm remains to be determined.

3.3.5. Effects of NC Dephosphorylation On Nucleic Acid Binding Potential.

Previous evidence with the P56 protein of CV A59 and JHMV has indicated the nucleic acid binding potential of this protein (Robbins et al., 1986; Beushausen et al., 1987). We undertook in this study to determine the effects of NC dephosphorylation on its ability to bind nucleic acids in vitro. The question of nucleic acid binding potential following dephosphorylation has significance in trying to

Figure 3.5. Effects of incubating NC with whole cell homogenate or the endosomal fraction on modification of p56, examined by Western blotting. Following reaction with Mab against NC the IgG was localized by the binding of I^{125}-protein A conjugates and autoradiography. In A, lanes 1,4, purified NC; lane 2, after dephosphorylation with endosome PPPase; lane 3, as in 2 with addition of 3 ug R_1; lane 5, NC following incubation with L cell homogenate. In B, lane 1, purified NC; lane 2, NC following incubation with WF rat brain homogenate. Native NC protein has a MW of approximately 56 KDa.


1
2
3
4
5
A
1
2
B

determine the sequence of events occurring during viral uncoating within the endosome and in helping to locate possible sites for interfering with the normal uncoating process.

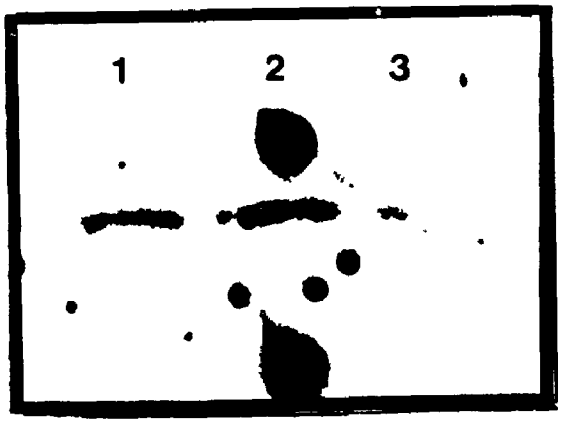
Following dephosphorylation of NC protein with endosome PPPase there is a large decrease in the ability of NC protein to bind labeled nucleic acids (Fig. 3.6). This evidence appears to indicate that dephosphorylation of P56 is in itself sufficient to alter its ability to bind nucleic acid independent of proteolytic processing.

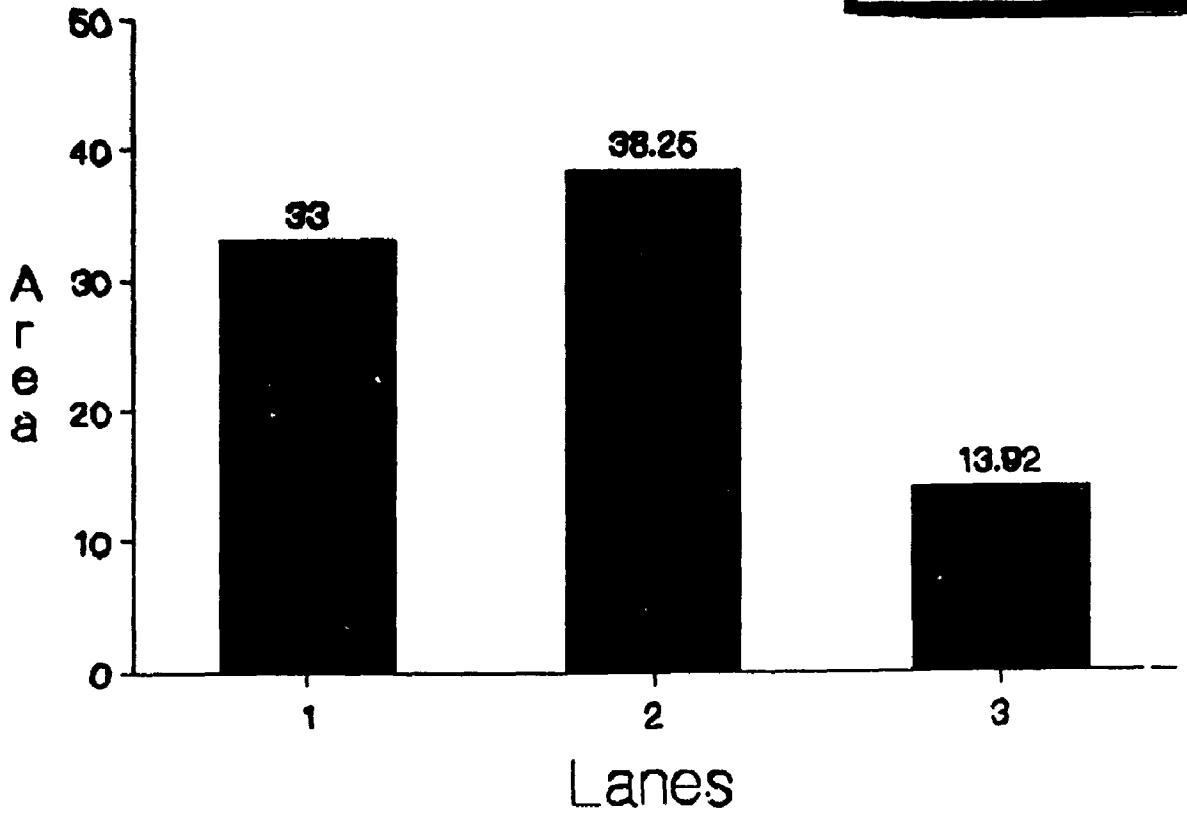
3.3.6. Protein Kinase Assay.

To assay for residual catalytic activity following separation of holoenzyme components on R_1 affinity columns, protein kinase assays were employed. These assays give an indication as to purity of R_1 preparations since contaminating catalytic component will phosphorylate the histone substrate while R_1 components give a base line response.

Protein kinase assays indicate good separation of PK components with R_1 subunit preparations showing no PK activity as compared to holoenzyme samples (data not shown).

Figure 3.6. Effect of NC dephosphorylation on binding of [^{32}P]-labeled plasmid g344 DNA demonstrated by a nucleic acid protein binding overlay assay. Inset, autoradiogram of nitrocellulose blot; lanes 1, 2, two purified preparations of NC protein; lane 3, NC following incubation with the endosomal fraction PPPase. Relative density of the bands was determined from the autoradiogram by a Ultrascan XL densitometer and expressed as the area per mm^2 occupied by each band.


1
2
3


50
40
30
20
10
0
Area
33
38.25
13.92
1
2
3
Lanes

3.4. DISCUSSION.

The age-related insusceptibility of glial cells from rodent hosts studied previously in this laboratory both in vivo (Sorensen et al., 1980) and in vitro (Beushausen and Dales, 1985; Wilson et al., 1986) is one of the most interesting and challenging mechanisms controlling replication of this virus.

Among the parameters thought to be involved in induction of glial cell differentiation and hence induction of JHMV resistance, is the stimulation of protein kinases and in particular cAMP dependent PK. Evidence was presented in this study showing the direct involvement of protein kinases in the previously observed onset of glial cell resistance to JHMV. Specific inhibitors of PK were successful in maintaining a permissive environment for JHMV replication in cultures concomitantly treated with dbcAMP. The maintenance of a permissive environment within glial cells was dbcAMP dose-dependent and varied with the affinity of PK inhibitor for cAMP dependent PK.

The inhibitor H8 was not however completely successful in preventing the induction of R_1 protein previously shown to be associated with in vitro oligodendrocyte differentiation (Beushausen et al., 1987). It is conceivable that the concentration of H8 used was not great enough to account for the higher number of cells present in the mixed cultures. To be

more definitive additional experiments should be done under conditions comparable to the experiments with isolated oligodendrocytes which demonstrated reversal of JHMV inhibition with PK inhibitor treatment. While these inhibitor studies cannot identify which specific PK(s) are involved we know from previous studies (Beushausen et al., 1987) that PK C and cGMP dependent PK are probably not involved. Hence, the focus has been on the involvement of cAMP dependent PK in the inhibition of JHMV replication in differentiated glial cells.

Previous evidence regarding WF glial cell differentiation documented the induction of the regulatory component of cAMP dependent protein kinase type 1 in primary cultures of oligodendrocytes from rats following treatment with the metabolite dbcAMP (Beushausen et al., 1987). We have shown in this study a possible association between free R_1 and a previously identified endosomal PPPase (Mohandas and Dales, 1989) which specifically dephosphorylates the NC protein from CV-JHMV. The interaction of R_1 with endosome PPPase results in decreased dephosphorylation of NC protein from JHMV when analysed in vitro. Inhibition of endosome PPPase is specific for the R subunit, with enzyme activity not affected by either C subunit or undissociated PK_1 or PK_2 holoenzyme. The addition of cAMP to the reaction mixture only slightly enhances the

ability of R_1 to inhibit the PPPase but gives holoenzyme preparations inhibiting activity by initiating dissociation of PK into its constituative components resulting in release of free R subunits.

Inhibition of host-cell endosome PPPase may have relevance to controlling JHMV infection since CV particles adsorbing to cell surface receptors are believed to penetrate via clathrin coated pits into endosomes (David-Ferreiera and Manaker, 1965; Krzystyniak and Dupry, 1984; Mallucci, 1966; Mizzen et al., 1985; Coulter-Mackie et al., 1985). Thus, the early stages of CV infection, including dephosphorylation of NC protein within endosomes, provide a possible site for restricting or inhibiting the normal sequence of reactions necessary for initiation of viral infection.

Support for previous observations regarding inhibition of a PPPase from rabbit skeletal muscle by the R subunit from PK_2 (Jurgensen et al., 1985; Khatra et al., 1985) is provided here since R_2 from dissociated PK_2 holoenzyme preparations inhibited NC dephosphorylation using our endosome PPPase. Thus, the R subunits from both PK_1 and PK_2 may have an additional function besides inhibiting or controlling catalytic phosphorylating activity involving inhibition of cellular phosphoprotein phosphatases. These enzymes are known to be involved in the dephosphorylation of a vast

number of cellular proteins and therefore inhibition of PPPase's by R subunits may provide an exquisite mechanism for controlling the rate and extent of&a4608Hprotein phosphorylation within cells. Recent evidence has also indicated other possible functions for free regulatory subunits within cells involving regulation of cAMP induced gene transcription (Mellon et al., 1989; Simon et al., 1989). These studies have demonstrated the ability of increased levels of R subunits within host-cells to block cAMP enhanced transcription and cellular differentiation.

In addition to the PPPase inhibition studies, evidence was also presented detailing the possible fate of p56 protein within endosomes. Dephosphorylation of NC protein with endosome fractions shows a specific loss of [^{32}P]-signal but does not result in any change in MW or indicate presence of proteolytic breakdown products previously identified within infected cells (Cheley and Anderson, 1981; Coulter-Mackie et al., 1985). Similar experiments dephosphorylating NC protein with cellular extracts however, demonstrate not only dephosphorylation of p56 but also show evidence of related lower MW products. Thus, dephosphorylation of p56 is probably not sufficient for changes in MW and probably requires a protease present in cytoplasmic environment for degradation of NC protein. Whether dephosphorylation of NC protein is required before proteolytic digestion of

p56 can occur remains to be answered.

In addition, preliminary evidence indicates changes in the ability of NC protein to bind nucleic acids following dephosphorylation by PPPase. Thus, dephosphorylation of NC protein may result in a loss or decrease of nucleic acid binding properties as assayed in an in vitro nucleic acid binding assay. These findings indicate that dephosphorylation of p56 may be essential for NC protein release of viral genome thereby allowing primary translation within cytoplasmic environment. Supportive evidence for changes in nucleic acid binding avidity following nucleocapsid protein(s) dephosphorylation has previously been reported for both retroviruses (Leis et al., 1984), and Herpes Simplex Virus types 1 and 2 (Wilcox et al., 1980), and thus may play an important role in the initiation or control of virus replication within cells.

In conclusion the results in this study present a unique possible mechanism for understanding the basis of age-related resistance associated with CV in rodents. Induction of JHMV resistance, correlated with increases in R_1 protein in differentiated oligodendrocytes, may in part be related to inhibition of a PPPase found in the endosome fraction of cells. Inhibition of this PPPase may prevent some essential step from occurring during the early stages of CV infection, possibly involving dephosphorylation of NC protein, thereby blocking viral

replication.

The following two chapters in this thesis will examine a second situation involving control of CV replication within host cells from rodents involving restriction of CV infection in glial cells from SJL mice. Chapter 4 will present results characterizing the infectious process of several CV in isolated glial cells from SJL neonates while Chapter 5 will examine a possible mechanism accounting for the observed restriction of virus infection.

CHAPTER 4

REPLICATION OF CORONAVIRUSES IN PRIMARY GLIAL CULTURES FROM GENETICALLY RESISTANT AND SUSCEPTIBLE MICE

4.1. INTRODUCTION.

Coronavirus (CV) infections in rodents are subject to multifactorial regulation. Among the parameters shown to be involved are the virus serotype, species and genetic constitution of the host, age and developmental stage of the central nervous system at the time of challenge, and route of inoculation (LePrevost et al., 1975; Levy-Leblond et al., 1979; Sorensen et al., 1984, 1982, 1980; Stohlman and Frelinger, 1978). Thus SJL mice which are relatively resistant to CV's A59 and JHM virus (JHMV) are highly susceptible to mouse hepatitis virus type 3 (MHV_3). Resistance in this strain may, in part, become manifested in an age-related manner but is also inherent in cells explanted from neonate and young animals, among them macrophages, astrocytes, and neurons (Collins et al., 1983; Knobler et al., 1981, 1984). One explanation offered to account for resistance of SJL mice to CV is the paucity or absence of viral receptors on the target cell (Boyle et al., 1987). However, detailed analyses by crossbreeding of genetically defined JHMV-susceptible B10 and -resistant SJL mice (Stohlman and Frelinger, 1978) imply that lack of receptors per se cannot account for the low

susceptiblity.

The availability of routinely prepared explant cultures of glial cells from mouse brain (Wilson et al., 1987) prompted us to do an in vitro analysis of the CV infectious process in anticipation that an explanation may be forthcoming for the resistance associated with SJL mice.

4.2. MATERIALS AND METHODS.

4.2.1. Continuous Line of Cells.

L-2 mouse fibroblasts (Rothfels et al., 1959) were cultured as previously described (Chapter 2), except that in addition to Eagle minimal essential medium (Flow Laboratories, Inc., McLean, Va.), the complete medium (CM) contained 8% Nu-serum (Collaborative Research, Inc., Waltham, Mass.) plus 2% fetal bovine serum (Bocknek Laboratories).

4.2.2. Viruses.

CV strains A59, JHM, and MHV_3 were assayed as PFU per milliliter (ml) as previously outlined (Chapter 2). The viruses were propagated by inoculating subconfluent cultures of L-2 cells with a multiplicity of infection (MOI) of approximately 0.1 PFU per cell. After adsorption for 60 min. at 22 C, the unadsorbed inoculum was removed by washing three times with phosphate buffered saline (PBS), pH 7.2. Fresh CM was added, and the cultures were incubated at 37 C in a humidified atmosphere with 5% CO_2. When approximately 90 to 100% of the cells in the monolayer had been recruited into syncytia, the released extracellular virus was collected by initial centrifugation at 1,000 x g for 30 min to remove larger cell debris and then concentrated by centrifugation in an SW28 rotor of a Beckman centrifuge at 70,000 x g for 60 min 4 C. The pelleted virus obtained

was resuspended in a small volume of CM and again concentrated by centrifugation at 70,000 x g for 60 min 4 C through a cushion of 20% sucrose. The pelleted virus was resuspended in CM, divided into aliquots, and stored at -70 C. Isotopically labeled virus was obtained by carrying out the infection for 12 hr at 37 C in the presence of 5 uCi of [^{3}H] uridine (23.4 Ci/mmol; NEN-Du Pont) per ml.

4.2.3. Mice and Primary Glial Cultures.

Primary glial cultures from neonatal mice were prepared as previously outlined (Chapter 2) with minor modifications. Mixed cultures consisting of oligodendrocytes and astrocytes were obtained from dissociated cerebral hemispheres of 1- to 2-day-old mice by propagating in 35-mm culture dishes (Nunc) cells derived on average from 1.8 brains in basal modified Eagle medium plus 10% fetal calf serum (BME_{10}) (Gibco Laboratories).

Cultures enriched with respect to oligodendrocytes or astrocytes were prepared from the primary explants of mixed cells as previously described (Chapter 2). Cell types in primary cultures were characterized as before (Chapter 2) by indirect immunofluorescence to myelin basic protein (MBP) in oligodendrocytes and glial fibrillary acidic protein (GFAP) in astrocytes. Staining for nonspecific esterase determined the

frequency of macrophage-type cells in primary glial cultures. In our mixed cultures, oligodendrocytes and astrocytes predomimnated, with 0 to 3% cells staining with nonspecific esterase. Cultures enriched specifically for oligodendrocytes were >90% MBP positive, while astrocyte-enriched cultures were >95% GFAP positive. Breeding and crossbreeding were done in-house from stock CD.1 and SJL mice purchased from Jackson Laboratory, Bar Harbor, Maine.

4.2.4. Monitoring Virus Adsorption and Sequestration.

Glial cultures were incubated at 4 C for 10 min before being inoculated with [^{3}H] uridine-labeled CV at an MOI of 1 PFU per cell. After adsorption for 60 min on a continuous rocking platform, the unattached virus was removed by three consecutive washes with 1 ml of cold (4 C) PBS (pH 7.2). Cell-virus complexes were released by scraping off the cells into 1 ml of cold PBS with a teflon policeman. The cell-associated labeled virus was collected by precipitating proteins with 1 ml of ice cold 20% trichloroacetic acid (TCA) on ice for 1 hr. Resulting precipitate was then collected onto Whatman GF/C filters with several washes of 10% cold TCA. Filters were air dried following wash with 100% ethanol and counted with 3 mls scintilint in liquid scintillation counter.

Internalization of inoculum virus after warming to

37 C was quantitated at designated intervals by determination of cell-associated cpm following release of any remnant extra-cellular virus with proteinase K (Sigma). Briefly, cultures were washed three times with 1 ml PBS (pH 7.2), and subsequently treated with proteinase K (0.5 mg/ml) in PBS for 45 min 4 C on a rocking platform. Protease treatment was terminated by collecting cells following scrapping with teflon policeman and mixing with 9 ml of warm BME_{10}. The suspension was then centrifuged at 650 x g for 2 min to pellet cells. Cell pellets were washed 2 times with BME_{10} before collecting cell-associated virus by TCA precipitation as outlined above.

4.2.5. Infectious Centers Assay.

Efficiency of CV association with host cells was also determined by infectious centers (ICT) assay. Briefly, the inoculum was adsorbed for 60 min at 22 C and allowed to penetrate during incubation for 90 min at 37 C prior to removal of the extracellular inoculum by proteinase K treatment (see section 4.2.4.). Samples were taken immediately after penetration (time zero) and after further incubation at 37 C for 24 and 48 hrs. After release, the glial cells were separated into monodisperse suspensions, which could be serially diluted, mixed with 10^6 L-2 cells, and plated onto 35-mm dishes. Upon attachment for 2 hr 37 C, the mixture of

L-2 and glial cells was overlaid with 0.5% methylcellulose in CM and incubated at 37 C for 24 hr to allow development of plaques. The plaques were enumerated on fixed and stained monolayers (Chapter 2).

4.2.6. Determination of Virus Antigens.

Expression of virus antigens in glial cells was monitored by immunoperoxidase staining. For this purpose, cultures propagated on 12-mm-diameter glass coverslips were infected and sampled as described in Results. Fixation with acetone for 2 min at 22 C permeated the cells while preserving antigenic reactivity.

Specific labeling of CV antigens was carried out using Mab to JHMV nucleocapsid protein in conjuction with anti-mouse immunoglobulin G Vectastain ABC kit (Vector Laboratories, Inc) as briefly outlind below. Prior to Ab labeling cultures on glass coverslips were washed 20 min with PBS (pH 7.2) and then incubated at 22 C with mouse pre-immune serum (Vectastain) for 20 min. Excess blocking serum was removed and cultures incubated 30 min 22 C with Mab to JHMV nucleocapsid protein (2 ul stock in 80 u/PBS). Cells were washed for 10 min in PBS with 3 changes of solution before incubating with diluted biotinylated antibody solution (Vectastain) for 30 min 22 C. Cultures were washed 10 min in PBS and then endogenous peroxidase activity quenched by treating slides with

0.3% H_2O_2 in methanol for 30 min at 22 C. Cells were washed a further 10 min with PBS and cultures were then treated with Vectastain ABC reagent containing Avidin DH and biotinylated horseradish peroxidase H for 60 min at 22 C. Cells were washed 10 min with PBS and incubated for 5 min 22 C with peroxidase substrate solution containing 0.01 % H_2O_2 and 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB). Coverslips were then washed five times with 2 mls H_2O and then let rinse 5 min in running tap water. Cells were counterstained with haematoxylin for 5 min, washed 5 min in H_2O, dehydrated in alcohol and xylenes and then mounted with DPX mounting solution.

4.2.7. RNA Extraction.

Cultures of glial cells in 35 mm tissue culture dishes, infected with CV or controls, were harvested for RNA extraction according to Cheley and Anderson (1984) with slight modifications. Monolayers were rinsed with 2 ml PBS and cells harvested by incubating cultures with 1 ml of 7.6 M guanidine-HCl in 0.1 M potassium acetate (pH 5.0) for 10 min at 37 C. Cell lysate was scrapped off with teflon policeman and passed ten times through 21 gauge needle on 1 ml syringe. Lysate was then transferred to 3 ml plastic tube mixed with 0.6 ml at -20 C 95% ethanol and incubated 18 hr at -20 C. The RNA was pelleted by centrifugation at 5,000 x g for 20 min at 4 C. The pellets were resuspended in 500 ul of 1:1 15% formaldehyde:20X

standard saline citrate (SSC), containing 3 M NaCl and 0.3 M trisodium citrate and stored -70 C until use.

4.2.8. Preparation of [^{32}P]-Complementary DNA to JHMV RNA.

Synthesis of complementary DNA (cDNA) was performed essentially as previously described by Coulter-Mackie et al. (1980) using JHMV-RNA as template (generously supplied by Dr. O. Sorensen). Reactions typically contained 0.5 ug JHMV-RNA, 10 ug calf-thymus DNA primers, 4 ul of 1 mg/ml actinomycin D (Calbiochem), 50 units AMV reverse transcriptase (Life Sciences), 100 uCi [^{32}P]-dcTP (400 Ci/mmole; Amersham), and 5 ul of salt-nucleotide buffer mix containing 0.25 M Tris-HCl (pH 8.0), 40 mM $MgCl_2$, 1.2 mM each of dATP, dGTP, dTTP, 0.25M KCl and 10 mM dithriothreitol (DTT). Components were mixed well and incubated 45 min at 37 C before addition of 0.5 ul of 3 mM dCTP (unlabeled) and reaction incubated further 15 min 37 C. The reaction mixture was then treated for 2 hr at 37 C with 13 ul of 2 M HCl. Labeled cDNA was isolated from remaining nucleotides by applying mixture to G-75 Sephadex column (45 cm X 1 cm) which was pre-equilibrated with buffer containing 0.3 M NaCl, 0.02 M Tris-HCl (pH 7.4), 3 mM EDTA and 0.1% SDS. Peak radioactive fractions were pooled, mixed with 100 ug yeast tRNA (Sigma) and cDNA precipitated by incubating mixture 18 hr at -20 C with 2 volumes of 95% ethanol. cDNA was pelleted by centrifugation at 16,000 x g for 60

min at -10 C followed by annealing pelleted cDNA with approximately 500 ug of uninfected total L-2 cell RNA in 0.6 M NaCl for 45 min at 68 C to remove any non-specific sequences complementary to host-cell DNA. The reaction mixture was equilibrated to 0.12 M sodium phosphate (pH 6.8) and single stranded cDNA isolated by separation on hydroxyapatite column at 60 C with 0.12 M sodium phosphate buffer (pH 6.8). Peak fractions were collected, pooled and cDNA precipitated 18 hr with 2 volumes 95% ethanol (-20 C) plus 100 ug yeast tRNA. The cDNA was pelleted by centrifugation at 16,000 x g 60 min at -10 C and then resuspended in small volume annealing buffer containing 50% v/v deionized formamide, 3X SSC, 1X Denhardt's solution (0.02% BSA, 0.02% polyvinyl pyrolindone 360, 0.02% Ficoll), 1 mg/ml yeast tRNA, 100 ug/ml alkali-sheared salmon sperm single stranded DNA and 0.01 M Hepes (pH 7.4). The cDNA was stored at -20 C until use.

4.2.9. Dot-Blot Hybridization.

Prior to dot-blot hybridization standardized quantities of total cellular RNA isolated from infected glial cultures were heated at 50 C for 15 min followed by quenching on ice. Samples were then applied to a Schleicher and Schuell Minifold dot-blot apparatus containing 0.45 um nitrocellulose (Millipore) on top of 2 layers of thick Whatman filter paper all of which had been pre-equilibrated with 10X SSC. Nitrocellulose was

subsequently baked 1 hr at 80 C in vacuum oven and then stored at 4 C until cDNA hybridization. Prior to labeling with cDNA probe the nitrocellulose sheets were pre-annealed for 18 hr at 39.5 C in buffer containing 50% formamide, 1 mg/ml yeast tRNA, 200 ug/ml salmon sperm DNA, 3X SSC and 1X Denhardt's buffer. cDNA hybridization was initiated following pre-annealing stage by incubating nitrocellulose sheets with approximately 1-2 x 10^6 cpm of [^{32}P]-cDNA for 48 hr at 42.5 C. Blots were then washed 5 X 5 min with 3X SSC at 22 C followed by 4 X 15 min washes with 0.1X SSC plus 0.1% SDS at 50 C. Finally, 5 X 1 min washes with 0.1X SSC at 22 C removed the SDS, blots were dried on filter paper and exposed for autoradiography at -70 C with Kodak X-Omat X-ray film.

4.3. RESULTS.

4.3.1. Virus Replication in Glial Cells from Susceptible, Resistant, and Hybrid Mouse Strains.

Intracerebral (ic) inoculation of the viscerotropic MHV_3 strain into either CD.1 or SJL/J mice causes a rapid, fatal encephalomyelitis (unpublished data). By contrast, SJL/J mice are highly resistant to ic infection with the neurotropic JHMV (Knobler et al., 1981; Stohlman and Frelinger, 1978), whereas CD.1 mice develop a central nervous system (CNS) disease (unpublished data). To test whether the appropriate match between host and virus strain associated with CNS disease susceptibility could be reproduced in vitro, cultures of mixed glial cells or cultures enriched for oligodendrocytes or astrocytes were challenged with MHV_3, JHMV, or A59 virus and monitored at intervals for the amount of PFU released into the supernatant. MHV_3 was replicated to high titers irrespective of the glial cell type or strain of mouse (Table 4.1). Data on the time course of virus production (Table 4.2) reveal that MHV_3 was produced efficiently, so that maximum titers were evident within 48 hr in SJL cells and 72 hr in CD.1 cells. No further increase in MHV_3 PFU occurred thereafter, undoubtedly because of extensive lysis of the massive syncytia which had formed. By contrast, production of JHMV was restricted in the SJL mixed glial

Table 4.1

Replication of Coronaviruses in Oligodendrocytes and Astrocytes from Resistant and Susceptible Mice:

mouse strain from which cultures originated	MOI[b]	Titer x 10^2 PFU/ml[a]			
		Oligodendrocytes		Astrocytes	
		JHMV	MHV_3	JHMV	MHV_3
SJL	1	11	3400	5	750
	10	49	2100	ND[c]	ND
CD.1	1	180	180	1950	3000
	10	390	575	ND	ND

a) values shown are averages from duplicate cultures sampled 24 hr after inoculation.

b) multiplicity of infection.

c) not done

Table 4.2

Comparison of Coronavirus Replication in Glial Cultures[a] From Pure Bred and Hybrid Mice:

Mouse strain from which cultures originated	Time after infection (hr)	Titer x 10^2 PFU/ml[b] JHMV[c]	MHV_3	A59
SJL	12	0.17±0.29(3)	590±114(3)	6±2(6)
	24	24±12(12)	435±292(14)	13±0.5(6)
	48	48±20(14)	886±62(6)	2±0.6(3)
	72	22±9(12)	578±119(6)	ND[d]
CD.1	12	350±216(5)	77±12(4)	ND
	24	3260±2110(13)	778±30(11)	21300±19600
	48	9440±500(9)	3340±2300(7)	95800±2700(3)
(CD.1 x SJL) F1	24	961±34(5)	1120±730(5)	ND
	48	6260±4320(5)	2400±2000(5)	ND
	72	943[e]±480(5)	213±52(5)	ND

a) primarily oligodendrocytes and astrocytes

b) the values are means with standard deviations. The number of cultures tested is shown in parentheses.

c) MOI 1 PFU/cell

d) not determined

e) decrease in titers was attributed to rapid cell killing

and enriched SJL oligodendrocytic or astrocytic cultures (Tables 4.1 and 4.2). The SJL restriction was evident as early as 12 hr after inoculation (Table 4.2). The highest JHMV titers, recorded within 24 h, did not increase further during the subsequent 48 hr and were lower by more than 2 orders of magnitude than with MHV_3. Inoculation with 10-fold-greater MOIs appeared to have only a small influence on JHMV titers in SJL cells (Table 4.1). In addition there was a restriction over replication of A59 in SJL but not in CD.1 cells. corresponding to that observed with JHMV (Table 4.2). However, with A59 the cytopathic effect was different from that evident with JHMV or MHV_3, because no formation of syncytia was detected (data not shown), confirming earlier observations by others on permissive A59 infection of C57BL/6 primary glial cultures (Lavi et al., 1987). Our results on infection with A59 also demonstrated that cell-cell fusion and syncytium formation are not a prerequisite for the deficient replication of some CVs.

Since there was no appreciable difference in virus yields from mixed glial as compared with oligodendrocytic or astrocytic cultures, in all subsequent experiments we used mixed cultures.

To test whether restriction over JHMV replication in SJL cells was a geneticlly dominant or recessive trait, we compared virus produced in glial cells from

susceptible, resistant, and F1 hybrid mice. The F1 hybrids were bred from matings of CD.1 females with SJL males. A comparison of JHMV and MHV_3 yields (PFU per ml) in CD.1, SJL, and (CD.1 x SJL)F1 cultures (Table 4.2) clearly revealed that glial cells from hybrid and CD.1 mice were equally permissive for JHMV, thus demonstrating that resistance is recessive. The decline in virus titers evident between 48 and 72 hr in Table 4.2 was attributed to rapid cell lysis, which could be observed by daily microscopic examination.

4.3.2. Early Events During CV-Glial Cell Interactions.

To determine whether the SJL restriction occurs during an early stage of cell-virus interaction, we examined the efficiency of JHMV and MHV_3 adsorption and uptake by glial cells from resistant and susceptible mice.

Cultures were inoculated at 4 C for 60 min with [^{3}H] uridine-labeled virus, and cell-associated cpm determined. The results (Table 4.3) revealed that about equal quantities of inoculum were adsorbed to glial cells from SJL and CD.1 mice. These data indicated that SJL restriction over JHMV is not likely to be due to an absence or paucity of viral receptors on host-cells.

The next stage of virus-glial cell interaction examined was sequestration of the inoculum. The rate of uptake, expressed as the percentage of cpm cleared from

Table 4.3

Adsorption of Inoculum Coronaviruses Labeled with [^{3}H]-Uridine:

Mouse Strain	Coronavirus Strain			
	JHMV		MHV_3	
	Total cpm added[b]	% initial cpm adsorbed	Total cpm added[b]	% initial cpm adsorbed
SJL	2300-8500	22 ± 2(6)	2200-19000	11 ± 1(5)
CD.1	2200-7200	23 ± 2(6)	2100-18000	10 ± 1(5)

a) all cultures were inoculated with MOI of 1 PFU/cell.

b) averages and standard deviations. Number of samples tested in parentheses.

the surface, was about equal in SJL and CD.1 glial cultures (Table 4.4). Between 68 and 100% of the stably adsorbed inoculum was internalized within 3 hr (Table 4.4). Reduction in cell-associated cpm during the interval from the end of adsorption at 4 C and incubation at 37 C for 1 hr was most probably due to desorption of the inoculum, indicating that only a small fraction of the input virus remained firmly attached. By comparison, after adsorption of these agents to the highly efficient L-2 host cells, about 30% of the inoculum initiated stable attachments (data not shown).

4.3.3. Cell-To-Cell Spread of Virus.

Previous studies with SJL peritoneal macrophages infected with JHMV (Knobler et al., 1984) and continuous LM-K cells infected with A59 (Mizzen et al., 1983) have indicated a defect in the spread of these CVs from initial foci, thereby restricting infection. Since neither adsorption nor internalization appear to be the steps limiting JHMV replication in SJL glial cultures, we examined the possibility that virus dissemination was affected. For this purpose, cells from inoculated SJL and CD.1 glial cultures were analyzed for the frequency of ICT formation. After infection and incubation for 0, 24, and 48 hr at 37 C, adherent cells were released and assayed as described in Materials and Methods. Immediately after adsorption, CD.1 cells infected with

Table 4.4

Sequestration of [^{3}H]-Uridine-labeled Inoculum Coronaviruses in Glial Cells:

		Coronavirus Strain			
		JHMV		MHV_3	
Mouse strain	Time after inoculation (hr)	Total cell-associated virus cpm[a]	% of adsorbed cpm not removed by proteinase k	Total cell-associated virus cpm[a]	% of adsorbed cpm not removed by proteinase k
SJL	0	512	0	2143	0
	1[b]	121	62	256	66
	3[b]	85	69	246	100
CD.1	0	490	0	1855	0
	1[b]	104	68	245	65
	3[b]	74	78	246	93

a) values are averages from duplicate cultures which are representative of data obtained in additional experiments.

b) after adsorption at 4 C, cultures were washed and incubated at 37 C with BME_{10} for 1 or 3 hr.

either JHMV or MHV_3 became ICT about five times as frequently as SJL cells (Table 4.5). The incidence of ICT in CD.1 cultures infected with JHMV and MHV_3 was amplified about 20-fold during 48 h. Incidence of ICT after infection of SJL cells with MHV_3 increased about 50 times in 24 hr and 80 times in 48 h. By contrast, there was a marginal increase in the frequency of ICT in SJL cultures inoculated with JHMV during the 48 hr duration of the experiment (Table 4.5), indicating that SJL restriction may involve cell-to-cell spread of virus.

The above data were corroborated by determining at 12 and 24 hr post-infection the time-related differences in the frequency of cells positive for virus antigen. Results (Table 4.6) revealed a rise in the incidence of syncytia and CV antigen-positive cells in CD.1 cultures inoculated with MHV_3 or JHMV and in SJL cultures inoculated with MHV_3, but not in SJL cultures inoculated with JHMV. These observations, which are entirely consistent with the ICT data, also suggest that SJL restriction over JHMV is due to inability of this virus to spread between cells.

4.3.4. Expression of CV RNA in Glial Cells from Susceptible and Resistant Mice.

Because of the above evidence that JHMV is restricted in SJL cells at a step subsequent to

Table 4.5

Infectious Centers Assay of Glial Cells:

Mouse strain from which cultures	Time (hr) after inoculation	Infectious centers per 1.2×10^6 cells[a]	
		JHMV[b]	MHV_3[b]
SJL	0	153 ± 62(6)	124 ± 59(6)
	24	825 ± 572(9)	5932 ± 2262(5)
	48	538 ± 395(9)	> 10,000[c](4)
CD.1	0	871 ± 317(5)	394 ± 81(5)
	24	> 10,000(2)	1417 ± 439(3)
	48	> 10,000(2)	> 10,000(5)

a) Values are averages and standard deviations. Number of samples tested in parenthesis.

b) all cultures were inoculated with MOI of 1 PFU/cell

c) > = greater than

Table 4.6

Frequency of Glial Cells Containing Coronavirus Antigen:

Mouse strain which cultures originated[a]	Virus[b] Strain	Time (hr) Post - Inoculation	Percentage of Antigen positive cells	Frequency of Syncytia /10^3 cells[c]
CD.1	MHV_3	12	1.4 ± 0.5	1(2)
		24	15.7 ± 3.1	20(3)
CD.1	JHMV	12	2.2 ± 0.4	2(3)
		24	9.0 ± 2.3	13(4)
SJL	MHV_3	12	2.6 ± 0.8	1(4)
		24	13.3 ± 2.2	33(3)
SJL	JHMV	12	1.8 ± 0.7	2(2)
		24	0.7 ± 0.3	0(0)

a) cell density was approximately 3 x 10^5 cells per well

b) all cultures were inoculated with MOI 1 PFU/cell

c) average number of nuclei/syncytium in brackets

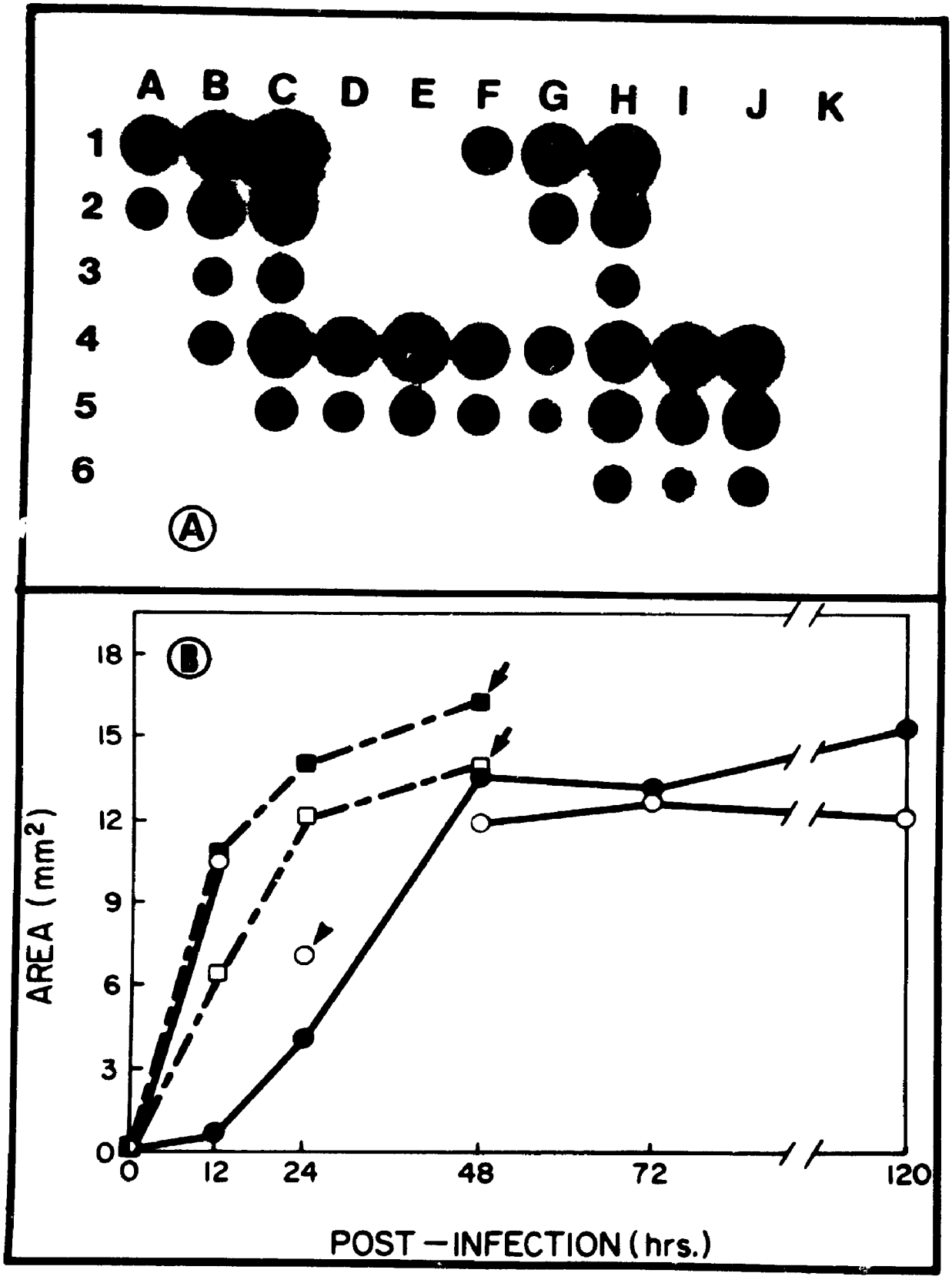
internalization, we investigated whether viral genome expression, in terms of RNA synthesis, might be affected. For this purpose, cells were sampled at intervals post-inoculation and the extracted RNA tested for viral specificity with cDNA probes in dot blots. The specific hybrids formed with the [^{32}P]-labeled probes were monitored in autoradiograms of the type illustrated in Fig. 4.1A. Data from densitometric scans of each dot blot in Fig. 4.1A, when expressed in terms of area (square millimeters) (Fig. 4.1B), provided a semi-quantitative assessment of CV-RNA. These results demonstrated that rates of RNA synthesis were approximately equal at 12, 24, and 48 hr after infection of CD.1 cells with JHMV or MHV_3 and SJL cells with MHV_3. By comparison, production of JHMV-RNA in SJL cultures was delayed, as evidenced by displacement of the points to the right in Fig. 4.1B. However, JHMV-RNA accumulated gradually in SJL cells so that by 72 to 120 hr post-inoculation the amount present was about the same as in the other three infections. While surprising, these data imply that the initial target cells continued to synthesize JHMV-RNA efficiently.

From these results, it is concluded that synthesis of JHMV-RNA in SJL glial cells is unlikely to be the rate-limiting step in the formation of infectious progeny.

Figure 4.1.A. Dot-blot analysis of CV-RNA present at various times after infection of mixed glial cultures. A-C: CD.1-JHMV RNA 12, 24 and 48 hr respectively post-infection (PI), at 1) 1:1; 2) 1:10; and, 3) 1:100 dilution. F-H: CD.1-MHV_3 RNA 12, 24 and 48 hrs PI at 1) 1:1, 2) 1:10; and, 3) 1:100 dilution. A-E: SJL-JHMV RNA 12, 24, 48, 72 and 120 hr PI at 4) 1:1; 5) 1:10 and, 6) 1:100 dilution. F-J: SJL-MHV_3 RNA 12, 24, 48, 72 and 120 hr PI at 4) 1:1, 5) 1:10; and 6) 1:100 dilution. I-K: RNA from uninfected CD.1, SJL and L cells, respectively at 1) 1:1; 2) 1:10; and, 3) 1:100 dilution.

4.1.B. Densitometric scans comparing dot-blot analyses of CV-specific RNA in CD.1 and SJL glial cell cultures infected with JHMV or MHV_3, illustrated in A. The concentrations of [^{32}P]-cDNA bound are expressed as the area in mm^2 occupied by individual dots.

Sampling terminated due to extensive CPE (Arrows). Abnormally low value (Arrowhead). ——o—— SJL-MHV_3 infection; ——●—— SJL-JHMV infection; -- -□ - — CD.1-MHV_3 infection; — -■- — CD.1-JHMV infection.


A B C D E F G H I J K
1
2
3
4
5
6
A
B
18
15
12
9
6
3
0
AREA (mm²)
0
12
24
48
72
120
POST – INFECTION (hrs.)

4.4. DISCUSSION.

The low efficiency of JHMV replication in primary glial cells from SJL mice, documented in this study, should be related to resistance of adult SJL mice to this agent (Knobler et al., 1981; Stohlman and Frelinger, 1978). On the basis of crossbreeding studies, resistance to JHMV in SJL mice has been postulated to be controlled by a single gene (Knobler et al., 1981) or by two factors (Stohlman and Frelinger, 1978). The ability to challenge glial cells in vitro with CVs enabled us to demonstrate unequivocally that strain-specific susceptibility of CD.1 mice and resistance of SJL mice is a genetic trait already inherent in neonatal cells. Since peritoneal macrophages (Knobler et al., 1981; Knobler et al., 1984; Stohlman and Frelinger, 1981), neurons (Knobler et al., 1981), and perhaps all potential host-cells in the body conform to the same constitutive control over virus replication, the age-related onset of SJL resistance must involve an independent, perhaps immune, mechanism, implying that two independent factors are controlling JHMV expression in vivo. The level of resistance may be greater with cells of non-glial origin, as evidenced by the lack of virus production previously reported with JHMV in SJL primary macrophages and neurons (Knobler et al., 1981; Knobler et al., 1984; Stohlman and Frelinger, 1981). In view of the very low contamination, if any,

by brain-derived macrophage cells in both CD.1 and SJL cultures, one can discount the influence of macrophages on suppression of JHMV replication, as observed when high concentrations of peritoneal macrophages were deliberately added to continuous cultures of glial cells (Stohlman and Frelinger, 1981).

Our genetic evidence showed that (i) closely related CV serotypes can either multiply efficiently, as found with MHV_3, or are greatly restricted, as is the case with JHMV and A59 and that (ii) based on data from hybrid F1 mice, susceptibility is a dominant trait, in line with results from previous in vitro studies (Collins et al., 1983; Knobler et al., 1984). From these findings one can assume that some cellular factor interacting with a viral component which is necessary for production of infectious particles is missing from SJL cells. In our systematic dissection of a sequence of steps in cell-virus interaction, analysis of adsorption showed that CV receptors for JHMV and MHV_3 are about equally abundant in CD.1 and SJL glial cells. These findings are supported by an ICT assay which showed that the frequency at which cells become infected initially is not determined by resistance or susceptibility of the mouse strain. Our data are therefore inconsistent with recent findings by others (Boyle et al., 1987) who concluded, on the basis of a solid-phase assay in which virions were bound to

isolated plasma membranes of hepatocytes and enterocytes fixed onto nitrocellulose sheets, that SJL restriction to CV-A59 is due to a lack of receptors on target cells. Our studies monitoring the uptake and sequestration of the inoculum indicated that this stage of the infection also proceeds with equal efficiency regardless of the virus type or strain of mouse from which glial cells are derived. Nor is the expression of viral RNA, assessed by dot-blots and immunomicroscopic detection of antigens, appreciably affected in the initial SJL cell targets of JHMV. Results from ICT assays and frequency of cells expressing CV antigen led us to conclude that low yields of JHMV in SJL cells are due to the inability of the progeny released from initially infected targets to spread the infection. This could be related to an absence of JHMV-induced syncytia from SJL cultures and their presence in CD.1 cultures. It should, however, be remembered that spread of virus and syncytiogenesis are not necessarily connected, as demonstrated with A59 virus.

In conclusion, the present study demonstrated the existence of a genetically regulated host-cell factor controlling CV-JHMV infection. The SJL glial cell restriction is CV strain specific and is not an age-related process as previously described with rodent oligodendrocytes (Chapters 2 and 3). Instead the restriction involves a late stage in the viral

replication cycle pertaining to dissemination of infection from initial foci. A possible mechanism for the imposed restriction will be presented in the following chapter describing the role of the E2 glycoprotein in CV infections.

CHAPTER 5

RESTRICTION OF JHMV INFECTION IN SJL GLIAL CELLS: ROLE OF THE E2 GLYCOPROTEIN

5.1. INTRODUCTION.

Our previous data contrasting CV-JHMV and MHV_3 infections with SJL glial cells (Wilson and Dales, 1988) demonstrated a possible defect in the intercellular spread or dissemination of JHMV infection. In CV the component which essentially controls the ability of virus to disseminate from initial target cells is the E2 glycoprotein. The E2 or peplomer protein is directly involved in cell-cell fusion, attachment, and infectiousness (Buchmeier et al., 1984; Collins et al., 1982; Dalziel et al., 1986; Talbot et al., 1984). Previous work has demonstrated that the infectiousness of certain viruses including orthomyxoviruses (Klenk et al., 1975; Lazarowitz and Choppin, 1975), paramyxoviruses (Scheid and Choppin, 1976, 1974) and CVs (Frana et al., 1985; Storz et al., 1981; Sturman et al., 1985 Yoshikura and Tejima, 1981), can be activated or enhanced through post-translational modifications of their respective peplomer proteins. Thus, bovine (Storz et al., 1981) and murine (Yoshikura and Tejima, 1981) CVs when treated with proteases exhibit enhanced syncytia formation and infectiousness.

Additional research employing variant CV containing

discrete changes in the peplomer proteins have also been shown to modulate the type and extent of infection (Fleming et al., 1986; Taguchi et al., 1988, 1986; Makino et al., 1983; Morris et al., 1989; Wege et al., 1988). While changes in E2 epitopes may result in reduced CV virulence (Dalziel et al., 1986; Fleming et al., 1986) they may also alter the type of disease from an acute encephalitis to a more chronic demylinating infection (Wege et al., 1988; Haspel et al., 1978; Dalziel et al., 1986).

These studies serve to illustrate the essential role the E2 glycoprotein plays in determining the course of infection with CV. The data in this study concern attempts to demonstrate a possible mechanism for the restriction of CV-JHMV replication in SJL glial cells involving a defect in the processing of the E2 glycoprotein. Experimental evidence involving post-translational modification of E2 glycoproteins by protease treatment and infections with a variant JHMV containing a truncated E2 glycoprotein will be discussed.

5.2. MATERIALS AND METHODS.

5.2.1. Continuous Line of Cells.

L-2 mouse fibroblasts (Rothfels et al., 1959) were propagated both in suspension and as monolayers at 37 C in complete medium (CM) containing Eagles minimal essential medium (MEM) (Flow laboratories, Inc.) supplemented with 8% Nu-serum (Collaborative Research), and 2% fetal bovine serum (Bocknek laboratories), as previously described (Chapter 2).

5.2.2. Primary Glial Cultures.

Mixed primary glial cultures from SJL mice consisting of oligodendrocytes and astrocytes were obtained by dissociating the cerebral hemispheres of 1-to-2 day old mice with tituration through 10 ml pipette and propagating the homogenate in 35-mm culture dishes (Nunc) (Chapter 4). On average each 35mm dish recieved 1.8 brains in basal modified Eagle medium (Gibco laboratories) containing 10% fetal calf serum (Bocknek laboratories), 10 ug/ml gentamycin (Gibco) and 0.6% dextrose, pH 7.2. Cultures were maintained at 37 C humidified atmosphere plus 5% CO_2 for 4 days before changing medium. Medium was then changed every 2 days until use.

5.2.3. Viruses.

CV strains JHMV and JHMV variant AT11fc were propagated by inoculating subconfluent cultures of L-2

cells at MOI of 0.1 PFU per cell. After adsorption for 60 min at 22 C, the unadsorbed inoculum was removed by washing three times with warm PBS (pH 7.2). Fresh CM was added, and cultures incubated at 37 C in humidified atmosphere with 5% CO_2. When approximately 90-100% of the cells in the monolayer had been recruited into syncytia, the released extracellular virus was collected by centrifugation at 1,000 x g for 30 min to remove large cell-debris. The suspension was then passed through 0.22 um filter to remove further particulate debris not pelleted by centrifugation. The virus suspension was subsequently aliquotted and frozen at -70 C until use.

JHMV variant AT11fc (obtained through collaboration with Dr. V. L. Morris, University of Western Ontario, Department of Microbiology/Immunology) was isolated following passage of wild-type JHMV (WT-JHMV) in CNS of Wistar Furth rats (Morris et al., 1989). The virus is characterized as having a truncated E2 glycoprotein and has a greater fusigenic phenotype as compared to their WT-JHMV. It should be mentioned that while AT11fc is more fusigenic and infectious than WT-JHMV it still retains the cell-type specific tropism for WF rat primary oligodendrocytes and not for WF astrocytes (Morris et al., 1989) thereby retaining this serotype related characteristic.

5.2.4. Enhancement of JHMV Infectivity With Protease Treatment.

Mixed glial cultures from SJL mice previously infected with JHMV were exposed to various proteases in a serum-free BME medium. Briefly, explanted cortical cultures from SJL mice were inoculated with JHMV and incubated for 4 hr at 37 C in BME_{10} medium. The medium was removed, and cultures washed twice in BME without serum before being treated with 0.1 ug/ml of trypsin, chymotrypsin or thermolysin (Sigma Chemical Co.) in serum-free medium for 10 hr at 37 C. At end of incubation cultures were washed twice with 1 ml BME_{10} and monitored at 37 C for PFU in the supernatant (Chapter 2) and frequency of cell-cell fusion.

5.2.5. Extraction of RNA.

RNA was extracted from infected or control L-2 cells using the Urea-LiCl extraction procedure of Auffray and Rougeon,(1980). Briefly, cells in monolayer were scraped off with teflon scraper into 2 mls cold PBS (pH 7.2) and pelleted by centrifugation at 1,000 x g for 10 min 4 C. The pellet was resuspended in 2.5 mls of 6M urea-3M LiCl solution and homogenized with 20 strokes in Dounce homogenizer on ice. The extract was incubated at 4 C for 18 hr and then centrifuged 8000 x g at 0 C. The pellet was mixed in 2.5 mls of 6M urea-3 M LiCl and the extraction repeated as above. Following the second

extraction the pellet was mixed with 2 ml 10 mM Tris-HCl (pH 7.5), 0.5 % SDS, 50 ug/ml proteinase K and incubated 20 min. at 37 C. The solution was then extracted twice with phenol followed by 2 chloroform extractions before addition of NaCl to 0.1 M final concentration. To this solution was then added 2.5 volumes of -20 C ethanol and mixture incubated at -20 C for 18 hr before final centrifugation of 8000 x g for 30 min 0 C to pellet RNA. The pellet was resuspended in a small volume of H_2O and stored at -20 C until use. Prior to agarose gel electrophoresis RNA was incubated at 50 C for 1 hr in sample buffer containing 14.5% glyoxal, 50% DMSO, 10mM Tris-acetate (pH 5.0), to denature RNA.

5.2.6. Northern Blot Transfer.

Purified RNA was analysed following agarose gel electrophoresis by northern-blot transfer according to Thomas (1980) and briefly outlined below. RNA was transferred from agarose gel to Nylon-66 paper (Fisher) by sandwiching the gel between filter paper and nylon-66 paper using 40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 7.4 (TAE) as buffer. The apparatus was incubated 18 hr at 22 C, the blot dried and stored at 22 C until use.

5.2.7. Agarose Gel Electrophoresis.

A 1.5% agarose (Gibco) solution in 10 mM NaPi (pH 7.0), was heated in microwave on high setting to

dissolve agarose. The agarose was then poured into protein gel aparatus (1 mm thick) and allowed to harden at 22 C 1 hr. Aliquots of denatured RNA were mixed with 1/3 volume 30% glycerol, 0.01% bromphenol blue and loaded onto gel. The sample was electrophoresed at 20 mA constant current for 5 hr with 10 mM NaPi (pH 7.0), as running buffer. The gel was then placed in 0.05 M NaOH for 30 min before equilibrating gel with 3 changes of TAE buffer.

5.2.8. Labeling and Purifying Cloned DNA.

Plasmid g34 Budzilowicz et al., 1985) generously provided by Dr. S. Weiss (University of Pennsylvania, Philadelphia, PA) contains a 1800-bp MHV-specific insert corresponding to the 5' end of nucleocapsid gene from CV-A59. DNA was labeled by nick translation essentially as described by Rigby et al., (1977) and outlined in Chapter 3. Unincorporated nucleotides were removed by centrifugation through Sephadex G-50 column as described by Maniatis et al., (1982) and outlined in Chapter 3.

5.2.9. Infection of Primary Glial Cells with CV.

Cultures of mixed glial cells, approximately 7-8 days post-explantation, were incubated at 22 C with 0.2 ml of CV isolates WT-JHMV and AT11fc, MOI of 1, for 1 hr with shaking. Following adsorption cultures were washed three times with 1 ml warm PBS and incubated at 37 C humidified atmosphere plus 5% CO_2 with 2 mls BME_{10}.

Cultures had medium changed daily with 2 mls BME_{10} following determination of released virus in supernatant by plaque assay (Chapter 2).

5.2.10. Infectious Centers Assay.

SJL glial cultures infected with JHMV were assayed for infectious centers following treatment with proteases. Briefly, infected cultures were plaque assayed 24 hr post-infection with JHMV and then treated for 12 hr at 37 C with 0.1 ug/ml trypsin in serum free BME. Cultures were washed three times with 2 ml BME_{10} and incubated at 37 C with fresh medium for 24 hr. Cells were again plaque assayed and then treated with 0.5 mg/ml proteinase k (Chapter 4) to remove extracellular virus. Glial cell cultures were then separated into monodisperse cultures, diluted and plated with L-2 indicator cells for infectious centers as previously outlined in Chapter 4.

5.3. RESULTS.

5.3.1. Infectiousness of JHMV in SJL Cells Following Protease Treatment.

Recently investigators have shown that proteolytic cleavage of the E2 glycoprotein of CV-A59 is associated with induction of Cl 17-1 cell-to-cell fusion activity (Frana et al., 1985; Sturman et al., 1985). To check the possibility that restriction on JHMV spread in SJL glial cells resulted from some deficiency in an exoprotease needed after synthesis of RNA, we treated infected glial cultures with trypsin, chymotrypsin, or thermolysin as previously outlined and monitored the cultures for PFU released into supernatant and induction of cell-cell fusion. A minor (about 25%), albeit reproducible, increase in PFU released into the supernatant was observed with trypsin and chymotrypsin, while thermolysin treatment had no enhancing affect on virus titer (Table 5.1). Microscopic examination of living and fixed cultures failed to reveal any increase in syncytia formation following protease treatment. Further studies also revealed no observable increase in the percentage of infectious centers (ICT) or PFU per infected cell in cultures treated with proteases (Table 5.2).

Additional experiments to show activation of viral dissemination after protease treatment of SJL glial

Table 5.1

Replication of JHMV in Primary Glial Cells

Following Protease Treatment:

Cultures derived from	time after treatment (hours)	% change in titer		
		trypsin	chymotrypsin	thermolysin
SJL	14[a]	22[b]	39	0
	24	15	23	-46
	48	37	17	-54

a) protease concentration of 0.1 ug/ml

b) values are averages from duplicate cultures

Table 5.2

Effects of Protease Treatment on Spread of JHMV[a] Infection:

mouse strain from which cultures originated	culture[b] conditions	infectious[c] centers per culture	% infected of total culture	PFU per infected cell
SJL	BME_{10}	787±301	0.07	6.1
	trypsin	538±194	0.05	6.2

a) MOI of 1.0

b) trypsin concentration of 0.1 ug/ml

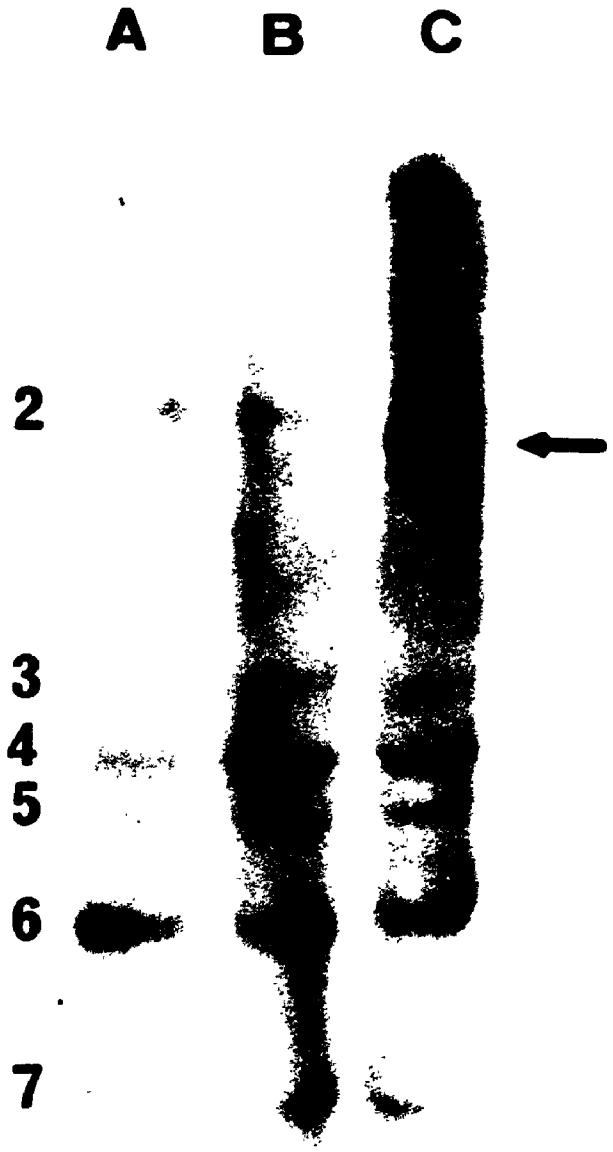
c) analysis completed 24 hours post-treatment with protease

cultures were also attempted. Experiments involving treatment of infected SJL cells with combinations of proteases as well as varying protease concentrations and exposure times all failed to enhance virus spread or release of PFU. Therefore, any putative SJL deficiency in protease activation of JHMV infectiousness remains to be demonstrated.

5.3.2. Northern Blot Analysis of WT-JHMV and AT11fc RNA.

Cultures of L-2 fibroblasts were infected with WT-JHMV and AT11fc at an MOI of 1.0 PFU per cell and infection allowed to proceed at 37 C until approximately 90% fusion. Cultures were then harvested for RNA extraction and CV specific mRNA analysed following northern blot transfer as previously described. Analyses of autoradiogram (Figure 5.1) show the only appreciable difference in mRNA pattern between WT-JHMV (lanes 1 and 2) and AT11fc (lane 3) appears to be in the truncated mRNA 3 coding for the E2 glycoprotein. The altered mRNA3 in AT11fc (lane 3) corresponds to approximately 2.85 X 10^6 Da compared with a 3 X 10^6 Da mRNA observed with WT-JHMV isolates in lanes 1 and 2. The truncated mRNA3, coding for the E2 glycoprotein, has previously been shown to result in a E2 peplomer protein of 165kDa in comparison to WT-JHMV which encodes a 180kDa protein (Morris et al., 1989).

Figure 5.1. Comparison of WT-JHMV and AT11fc mRNA by Northern blot analysis. SJL glial cultures were infected at an MOI of 1 and RNA extracted approximately 24 hrs PI. Lanes A and B, two isolates of WT-JHMV; lane C, JHMV variant AT11fc. Arrow shows mRNA difference between AT11fc and WT-JHMV patterns. Northern blots probed with [^{32}P]-labeled CV specific plasmid g344 as outlined in materials and methods. Numbers at side of blot correspond to CV specific mRNA species found in infected cells.


A
B
C
2
3
4
5
6
7

5.3.3. Replication of WT-JHMV and Variant AT11fc in Cultures of Mixed Glial Cells.

Cultures of mixed glial cells from resistant SJL mice and susceptible CD.1 mice were infected with either WT-JHMV or variant AT11fc to determine if the variant AT11fc, containing an altered E2 glycoprotein, could overcome the restriction in SJL cells observed with WT-JHMV. Cultures were monitored for 72 hr post-infection for both fusion induction and release of PFU into supernatant (Table 5.3).

Infection of SJL cultures with WT-JHMV demonstrates the restriction in SJL glial cells with low amounts of virus being produced in the absence of syncytia formation. In contrast, CD.1 cultures infected with WT-JHMV show a highly productive infection with massive fusion of the monolayer. Identical cultures, when challenged with the variant AT11fc however demonstrate a slightly different pattern of infection (Table 5.3). While CD.1 cultures show a highly productive infection with massive fusion and high virus titers analagous to the infection observed with WT-JHMV, AT11fc appears to be slightly more efficient with maximum titers being reached by 48 hr post-infection compared with 72 hr for WT-JHMV. Cultures of glial cells from SJL mice infected with AT11fc however, show no similarity to counterparts infected with WT-JHMV. AT11fc infection results in much greater release of virus, usually at least 2 logs more

Table 5.3

Replication of WT-JHMV and Variant AT11fc in Glial Cells[a] From Resistant and Susceptible Mice:

cultures derived from	time after incubation (hours)	Titer (X 10^2 pfu/ml) JHMV[b]	AT11fc[b]
SJL	24	4.6 ± 0.15(5)[c]	825 ± 110(5)
	48	3.9 ± 2.1(5)	1,080 ± 350(5)
	72	10.4 ± 4.3(5)	1,320 ± 640(5)
CD.1	24	375[d]	9,000
	48	16,000	39,000
	72	25,000	390

a) Primarily oligodendrocytes and astrocytes

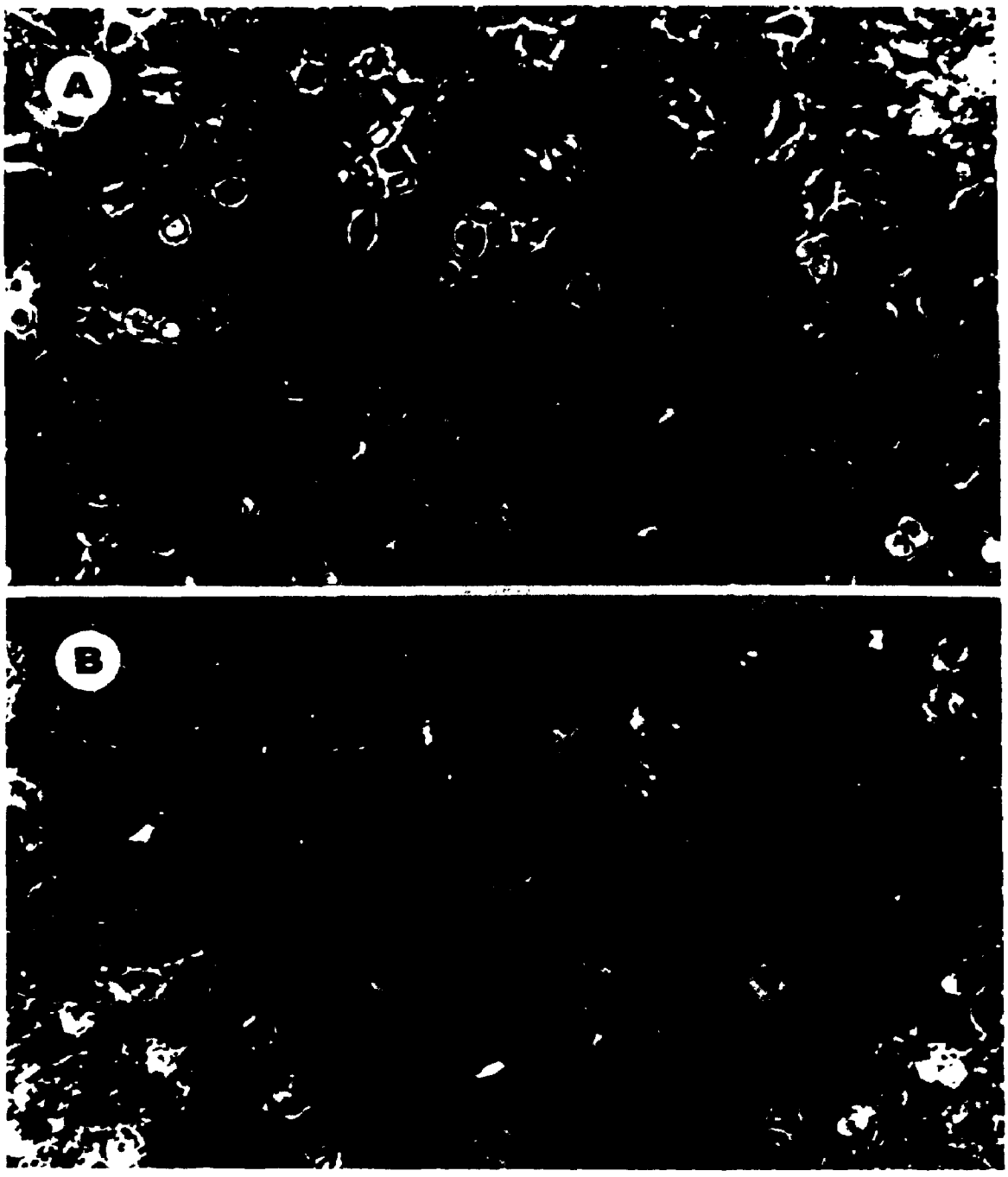
b) MOI, 1 PFU per cell

c) Values are means with standard deviations. The number of cultures tested is shown in parenthesis.

d) Values are means of duplicate samples.

virus, with the addition of glial-cell fusion induction (Figure 5.2) commencing as early as 24 hr post-infection. Thus, the variant AT11fc, containing a modified or truncated E2 glycoprotein, appears capable of overcoming, at least partially, the restriction imposed on WT-JHMV replication in SJL glial cells.

Figure 5.2. Phase contrast images of SJL glial cultures 24 hrs post-infection with WT-JHMV and variant AT11fc at an MOI of 1. In A, SJL mixed culture infected with WT-JHMV. In B, identical culture infected with AT11fc. Arrow denotes large area of cell-cell fusion X 600.


A
B

5.4. DISCUSSION.

In vitro challenge of SJL glial cells with CVs JHMV and MHV_3 illustrates the complex interaction of virus and host in determining disease potential. The highly serorelated strains show quite distinct results in SJL glial cells with MHV_3 giving high titers of virus with fusion of cells while JHMV infection gives low titers and little or no fusion (Wilson and Dales, 1988). One possible explanation for these observations involves differences in the E2 glycoproteins of these viruses. The E2 glycoprotein, responsible for both attachment to and fusion of host-cells (Collins et al., 1982), exhibits a high degree of variability among CV strains (Talbot and Buchmeier, 1985; Schmidt et al., 1987; Fleming et al., 1983: Luytjes et al., 1987). The importance of this protein in determining the outcome of virus challenge is underscored by research showing that site-specific changes in the E2 protein can result in drastic changes in the type and extent of viral pathology (Dalziel et al., 1986). At least 5 distinct regions on the E2 protein have been identified (Dalziel et al., 1986; Wege et al., 1988) with different regions possibly controlling various aspects of this proteins function. Thus the manner with which the host-cell manufactures, transports and processes the peplomer proteins from different CVs will regulate the type and extent of infection (Frana et al., 1985; Sturman et al.,

1985).

One of the most important post-translational modifications of the peplomer protein may involve processing of the protein from the 180K form to two 90K subunits (Sturman et al., 1985). This host-cell protease dependent processing of E2 glycoprotein has previously been shown to enhance CV infectivity and spread (Storz et al., 1981; Yoshikura and Tejima, 1981; Sturman et al., 1985). Results from this study however, with exogenous addition of proteases failed to bring about enhanced PFU release or fusion induction. Thus, if JHMV is restricted in SJL cells because of a lack or deficient E2 proteolytic processing our results provide inadequate data to support such a claim. Encouraging results were however obtained when SJL cultures were infected with the JHMV variant AT11fc. Infection with AT11fc resulted in a large increase in PFU released and was also associated with massive fusion of SJL cells analogous to infections with MHV_3 documented in the previous Chapter. Thus, AT11fc which contains a truncated E2 glycoprotein appears to overcome the restriction imposed on WT-JHMV. This deletion in AT11fc, corresponding to approximately 90 amino acids, may result in a E2 conformational change which is favourable for proteolytic cleavage and activation of cell-fusing potential in SJL glial cells.

In addition to these in vitro studies we hope in

the near future to use the variant JHMV to address the issue of whether a single gene (Knobler et al., 1984) or two factors (Stohlman and Frelinger, 1978), possibly involving an immune component, are controlling the resistance of SJL mice to in vivo challenge with JHMV. Pre-weanling and adult SJL mice will be challenged with both WT-JHMV and the variant AT11fc to determine if the induction of cell-cell fusion by the variant can overcome the imposed restriction on neurological disease production. Based on our in vitro observations we would predict that the dissemination of infection from initially infected foci, analagous to infections in permissive hosts, would result in disease production unless some immune component is involved in successfully clearing the virus from the nervous system.

In conclusion, this study demonstrates the complex interaction of the host-cell and virus in determining the outcome of initial virus challenge. With SJL glial cells in vitro, the restriction imposed on WT-JHMV infection may be related to the inability of the host-cell to adequately process the E2 glycoprotein thereby restricting the spread of virus infection from initial infected foci.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The neurotropic potential of some CVs and the possible relevance to human demyelinating conditions underscore the importance of determining the outcome of virus challenge within the nervous system and in defining those parameters which control virus replication. To this end our research has focused on characterising the interaction of CVs with cells of glial origin and subsequently defining the possible mechanisims responsible for restricting virus replication. The studies within this thesis documented two separate situations involving CV-glial cell interactions including i) the age-related acquisition of glial cell resistance to CV-JHMV infection, and ii) the inherent resistance to CV challenge found in glial cells from specific host-genotypes. This chapter will briefly summarize the research findings and suggest possible future experimental directions.

6.1. INFLUENCE OF GLIAL CELL DIFFERENTIATION ON CORONAVIRUS INFECTION.

The establishment of in vitro primary cerebral explant cultures, containing oligodendrocytes and astrocytes, from mice enabled us to identify and better characterize the CV infectious process in cells of murine origin. Upon challenge with either JHMV or MHV_3

no discrimination in cellular tropism was evident with both viruses replicating in either cell type. These results on murine glial cell infectability correlate with in vivo findings concerning susceptibility of rodent hosts to these CV agents (Sorensen et al., 1982; Hirano et al., 1981; Stohlman and Weiner, 1981). In addition, the induction of glial cell differentiation, through treatment of cultures with the metabolite dbcAMP, resulted in a profound suppression of CV replication in oligodendrocytes and to a lesser extent in astrocytes. These findings, consistent with previous studies by Beushausen and Dales, (1985), focus further on the age-related acquisition of resistance to CV challenge occurring in both rats and mice, and led us to attempt to define a possible mechanism which might account for these observations.

One possible mechanism involved the inhibition of a phosphoserine phosphoprotein phosphatase (PPPase) found in the endosome fraction of host-cells. The inhibition of this enzyme, previously shown to dephosphorylate the NC protein of JHMV, may interupt the normal sequence of events occurring during the early stages of CV infection. Evidence was presented showing the inhibition of an endosomal PPPase with regulatory subunits of cAMP-dependent protein kinases types 1 (PK_1) and 2 (PK_2). Inhibition of PPPase was specific for the regulatory subunits with no inhibition demonstrated when using

catalytic subunits or undissociated PK holoenzymes. The specific involvement of PK in development of host resistance to CV challenge was indicated through studies employing inhibitors of PK activities. Treatment of glial cultures with PK inhibitors maintained a permissive environment for CV replication even in the presence of dbcAMP.

Modulating the state of NC phosphorylation appeared to result in changes in its nucleic acid binding potential with dephosphorylation resulting in a large decrease in binding avidity. Additional evidence was presented showing that dephosphorylation of NC protein within host endosomes was not sufficient for changes in NC mw and that a component found in host-cell extracts, most likely a proteolytic component, was required for processing of NC protein to lower mw forms found in infected cells. This evidence taken together resulted in our hypothesis concerning the induction of JHMV resistance in differentiated oligodendrocytes. Onset of JHMV resistance, coincident with induction of R_1 from PK_1, involves the specific inhibition of an endosomal PPPase which normally dephosphorylates the NC protein of CV. Inhibition of this PPPase prevents some essential sequence of events from occurring within host endosomes possibly involving dephosphorylation of the NC protein and subsequent release of viral genomes for primary translation. This effectively results in blocking CV

infection at an early stage post-adsorption.

6.2. INHERENT RESISTANCE OF SJL GLIAL CELLS TO CV-JHMV.

The second situation involving control over CV replication within the nervous system studied in this thesis involves the serotype-specific inhibition of CV replication in glial cells derived from neonatal SJL mice. Mixed glial cultures, containing both oligodendrocytes and astrocytes, restricted the replication of CV-JHMV and A59 but fully supported the replication of the highly sero-related strain MHV_3. By comparing the infectious process of both JHMV and MHV_3 at various stages during the replication cycle we were able to demonstrate a block in JHMV infection involving the intercellular spread or dissemination of infection. Thus, the early events surrounding adsorption or uptake of virus from cell surface were not responsible for the observed restriction implying that host-cell receptors were not limiting or controlling JHMV infection as suggested by Boyle et al., (1987), for A59 infection in SJL enterocytes. Attempts to enhance JHMV infectiousness through exoprotease treatment of infected cultures failed to stimulate fusion induction but did result in a marginal increase in PFU release into culture supernatant. Additional studies with a variant JHMV (AT11fc), containing an altered E2 glycoprotein, were successful however in producing between 2-3 logs more virus than similar cultures infected with WT-JHMV.

Perhaps more importantly infection with AT11fc resulted in massive fusion of SJL monolayers analagous to infections in highly permissive CD.1 cultures. These results seem to imply that the imposed restriction on WT-JHMV might involve an inability of the host-cell to adequately process the E2 glycoprotein of the virus thus failing to activate its cell-to-cell fusigenic potential. This in turn restricts the spread of the virus from initially infected foci thereby effectively controlling the infection.

6.3. FUTURE DIRECTIONS AND CONCLUSIONS.

Studying the mechanisms controlling CV infection within cells from the nervous system has led to some very interesting and challenging insights. Our research on the age-related insusceptibility of glial cells to CV challenge has not only suggested a possible mechanism responsible for this restriction but also illustrates the complex interactions of cellular enzymes such as protein kinases and phosphatases in the normal maturation of these cells. Future studies should certainly concentrate on further characterising the infectious process within differentiated glial cells as many questions remain. What type and extent of specific interactions involving cellular PPPase and regulatory proteins are involved and how do these complexes interact with incoming virus particles during uncoating

are interesting areas for development. In addition the cell-type specific tropisms of JHMV and MHV_3 for glial cells in rats must be further examined to locate possible sites responsible for virus restriction.

Our additional studies on restriction of JHMV replication in SJL glial cells demonstrates the complex interactions of viral proteins and host-cells in determining outcome of virus challenge. The ability of host-cells to inadvertently control CV replication through inadequate synthesis or processing of viral peplomer proteins indicates that more work is necessary in defining those regions or protein conformations responsible for productive CV infections.

In conclusion, the studies within this thesis have further defined and characterized the infectious process occurring within host-cells from the nervous system of rodents and have hopefully shed insight into possible mechanisms responsible for control of CV replication within these cells.

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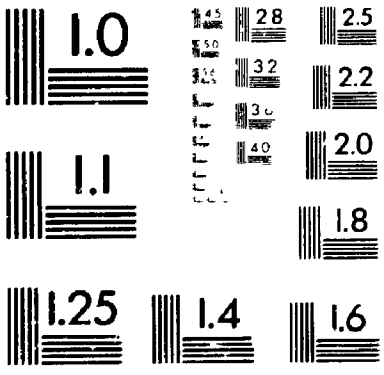

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